

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS



Ciências
ULisboa

**Impacts of *Acacia longifolia* invasion on soil nutrient cycles:
From invasion to solution**

“Documento Definitivo”

Doutoramento em Biodiversidade, Genética e Evolução

Florian Ulm

Tese orientada por:

Cristina Maria Filipe Máguas Silva Hanson

Cristina Maria Nobre Sobral de Vilhena da Cruz Houghton

Documento especialmente elaborado para a obtenção do grau de doutor

2019

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS



**Ciências
ULisboa**

**Impacts of *Acacia longifolia* invasion on soil nutrient cycles:
From invasion to solution**

Doutoramento em Biodiversidade, Genética e Evolução

Florian Ulm

Tese orientada por:

Cristina Maria Filipe Máguas Silva Hanson

Cristina Maria Nobre Sobral de Vilhena da Cruz Houghton

Júri:

Presidente:

• Doutora Maria Manuela Gomes Coelho de Noronha Trancoso, Professora Catedrática e Presidente do Departamento de Biologia Animal da Faculdade de Ciências, Universidade de Lisboa

Vogais:

- Doutor Bruce Arthur Osborne, Professor School of Biology and Environmental Science da University College Dublin (Irlanda);
- Doutor João José Pradinho Honrado, Professor Auxiliar Faculdade de Ciências da Universidade do Porto;
- Doutora Hélia Sofia Duarte Canas Marchante, Professora Adjunta Escola Superior Agrária do Instituto Politécnico de Coimbra;
- Doutor Luís Miguel Cortez Mesquita de Brito, Professor Coordenador Principal Escola Superior Agrária do Instituto Politécnico de Viana do Castelo;
- Doutora Cristina Maria Filipe Máguas da Silva Hanson, Professora Associada Faculdade de Ciências da Universidade de Lisboa (orientadora);
- Doutor Sergio Chozas Vinuesa, Bolseiro Pós-Doutoramento Faculdade de Ciências da Universidade de Lisboa.

Esta dissertação teve o apoio da Fundação para a Ciência e a Tecnologia (FCT) através da bolsa de doutoramento PD/BD/106061/2015 e teve apoio da União Europeia através das bolsas COST-STSM-ECOST-STSM-ES0806-010512-017110, COST-STSM-ECOST-STSM-ES0806-011012-023168 e FP7-PEOPLE- 2010-IRSES

Documento especialmente elaborado para a obtenção do grau de doutor

2019

Acknowledgements

This work would not have been possible without many people. However, nor will I be able to write down many of them here, neither will the order ever be right, because how can you thank from the top to the bottom of a page, when its really a net of people you love and care for that surrounds you in all aspects of life?

When I came to Portugal so many years ago, I thought I would stay only for some months and go back to Germany and who knows... if I wouldn't have met Sara, with her kindness, love and happiness, maybe I wouldn't be here anymore. So I want to thank her for being who she is, for helping me being a better self and for creating a future together with me.

Then, I wonder, if my family wouldn't have been here with me in Portugal for so many years before I came alone, maybe I wouldn't smell home when I come back here after a trip abroad. So I want to thank them for being who they are, for their unconditional love and help and for making me a curious person, as we should all be if we want to understand something about the wonders around us.

But the way that led me here was never straightforward, and it will also never be in the future. On the diffuse path taken through space and time, I had the opportunity and the gift to encounter so many great people, of which some I have the honour to call my friends. So I want to thank all of them for being who they are and for dancing, laughing, thinking, playing, planning and dreaming with me.

Surely, many paths lay in front of all of us, but sometimes it takes the right place and the right time for you to choose one of the many. Here in Lisbon I found this place and I remember well, when I first came to the university with my Erasmus paper in the hand and no idea what I wanted to do and found that both people that would guide, help and support me so much were sitting in two offices in the same corridor. So I thank my supervisors, that I can hope to call my friends as well for being who they are and for giving me the freedom I need to stay curious while leading me back on track when I go astray.

There is, though, one that needs to be thanked above anyone and anything else: life itself. Life, which is both the observation of this work and the observer himself. And, sadly, in a time when people look up to science when its useful for them and they dismiss it when its in their way, its often life itself that is loosing out. We are in a situation of existential crisis, a turning point of humanity. And if there is one thing we need to understand and care for, it is how we can help life to survive, be it only for the survival of our own kind.

This humble work wants to give an infinitely small contribution to this understanding, and while the wind, the sun, the rain and the green garden around us might not care if we know or we don't, maybe knowing something about them might help us to be grateful and become the gardeners of the world, not their destroyer. So I thank life, just for being what it is.

Resumo

Alterações antropogénicas dos ciclos biogeoquímicos, como os do azoto (N) e fósforo (P) tal como a dispersão de espécies fora dos seus habitats nativos, tornando-as invasoras em alguns casos, são alguns dos fatores que modificam profundamente os ecossistemas e o seu funcionamento. Os ecossistemas dunares oligotróficos, têm sido particularmente susceptíveis à instalação de espécies invasoras. Uma das espécies invasoras dos sistemas dunares mais importante em Portugal é a *Acacia longifolia*; uma espécie leguminosa que tem sido considerada um verdadeiro “engenheiro do ecossistema”, alterando completamente o funcionamento do solo e transformando ecossistemas biodiversos em sistemas monoespecíficos. No entanto algumas das alterações que provoca nestes ecossistemas, nomeadamente o aumento da quantidade de matéria orgânica (MO) no solo, actividade microbiana e a mineralização de P e N, podem até ter um efeito positivo no crescimento de espécies nativas contíguas. Este impacto no desenvolvimento da vegetação nativa só ocorre quando a abundância de *A. longifolia* é ainda muito baixa, nas fases iniciais do processo de invasão. Este e outros estudos, levantaram a hipótese de uma utilização da biomassa derivada das ações de controlo de *A. longifolia*, quando transformada num “composto verde”, em solos agrícolas arenosos pobres, aumentando a MO estável, a actividade microbiana, e a capacidade de retenção de água e nutrientes no solo. O trabalho aqui reportado pretende mostrar a exequibilidade desta visão: os capítulos 2 e 3 usam o impacto da *A. longifolia* nas folhas da espécie nativa *Corema album* como indicador bioló-

gico de alterações ao nível do solo, enquanto os capítulos 4 e 5 investigam o compostado da biomassa da *A. longifolia* como corretivo do solo para fins agrícolas.

A fase inicial da invasão de dunas primárias oligotróficas no sul de Portugal por *A. longifolia* foi estudada no capítulo 2. Foi medida a biomassa das copas de arbustos nativos de *C. album* invadidos e não invadidos, bem como a distribuição de matéria orgânica (MO) em várias fracções do solo. De acordo com a nossa hipótese, a presença de *A. longifolia* estaria relacionada com o enriquecimento foliar em ^{15}N de *C. album*, o qual é interpretado como evidência da transferência de azoto fixado pela leguminosa invasora para a planta nativa. Nas plantas de *C. album* invadidas, a biomassa das raízes foi maior, tal como a MO da fracção limo-argilosa do solo da rizosfera. Finalmente, verificou-se que a relação entre a MO no compartimento biótico e a MO no solo poderia ser um precoce indicador da invasão por *A. longifolia*.

No capítulo 3 comparou-se a biomassa presente em vários compartimentos do solo, a velocidade potencial dos ciclos de N, C e P tal como a estequiometria da MO de uma leguminosa nativa (*Stauracanthus spectabilis*) e da *A. longifolia*. Foi hipotetizado que a leguminosa invasora requer menos P por unidade de biomassa produzida e exibe maior potencial de aquisição de nutrientes, o que poderia alterar a relação N/P do solo rizosférico e da biomassa. Em comparação com *S. spectabilis*, as plantas de *A. longifolia* apresentaram maiores copas, maiores níveis de MO no solo sob a sua influência e concentrações mais baixas de

P nos seus tecidos. Além disso, a maior concentração de N na raiz e o aumento da actividade enzimática na rizosfera das plantas de *C. album* sob a influencia de *A. longifolia* estiveram associados à diminuição da concentração foliar de P. Estes resultados mostram que a *A. longifolia* faz um uso mais eficiente do P na produção de biomassa enquanto exerce um forte impacto na estequiometria N/P do solo e do sistema nativo.

No capítulo 4 avaliou-se o efeito da adição ao solo de um compostado somente de biomassa de *A. longifolia* (CO) e de um compostado de uma mistura de biomassa e folhada (CL) a um solo agrícola arenoso. O efeito foi avaliado na determinação das concentrações de nutrientes biodisponíveis (N e P) e pH no extracto do solo junto com a cinética das enzimas da mesma amostra. Para obter uma resolução temporal elevada foi explorado um novo método de alto rendimento em microplaca na determinação das concentrações de nutrientes e pH no extracto do solo junto com a cinética das enzimas da mesma amostra. Ambos os tipos de compostado, CO e CL, tiveram elevadas taxas de nitrificação mas também efeitos benéficos na qualidade do solo e imobilizaram N, potencialmente diminuindo a lixiviação de N. Uma vez que neste trabalho foram encontrados fortes efeitos de interação com P e N disponível, é possível prever uma alta capacidade de solubilização de P na folhada de *A. longifolia* em sistemas naturais, e um potencial para libertar N imobilizado na MO num ambiente agrícola com adição de P.

O uso de compostado da *A. longifolia*, junto com compostado de resíduos municipais (CM) para a produção de milho num ambiente urbano foi investigado no capítulo 5. Hipote-

tizando que as variedades de polinização aberta (VPAs) mantêm alto valor nutricional em condições de baixa fertilização, um híbrido comercial e uma VPA local de milho foram cultivadas em solo não fertilizado e com duas aplicações de compostado: só CM e um compostado de *A. longifolia* misturado com CM para criar um regime de fertilização média. As parcelas não fertilizadas exibiram baixa produção de grãos (1.9 t ha^{-1}), comparado com o compostado de *A. longifolia*/CM (6.1 t ha^{-1}) e somente CM (7.8 t ha^{-1}), que são taxas de produção comparável ao rendimento de milho sob fertilização inorgânica. Portanto, ambas as misturas de composto foram eficazes no aumento do rendimento de grãos em ambas as variedades de milho. Porém, a VPA produziu grão com maiores níveis de micronutrientes e menos metais pesados, então está mais bem adaptada à agricultura urbana sustentável baseada em compostagem.

Em resumo, a co-limitação de N e P necessita de ser considerada na invasão de ecossistemas oligotróficos. Além disso, os processos do solo precedem os efeitos acima do mesmo, tornando a remoção precoce de plantas jovens imperativa para a conservação do ecossistema. A biomassa compostada pode ser utilizada com segurança como corretivo do solo, com efeitos benéficos para vários parâmetros do solo e da planta, o que pode estimular a erradicação futura de povoamentos de *A. longifolia*.

Palavras-chave

Acacia longifolia, planta invasora, interação planta-solo, composto, estequiometria N/P

Abstract

Anthropogenic alterations of nutrient cycles and the global redistribution of plant species have a profound impact on soils and the ecosystems relying on them. Invasive woody legumes can engineer oligotrophic ecosystems towards increased biomass production and nutrient turnover. This increased biomass is detrimental to native ecosystems, but could also be useful as a compost feedstock for agricultural purposes.

The aim of this study was to observe soil changes after *Acacia longifolia* invasion in Portuguese dune systems, using *Corema album* foliar $\delta^{15}\text{N}$ as a tracer for invasion impact. It provides novel insight into initial invasion of nursery shrubs and changes in soil fractions. It also relates community-scale aboveground invasion impact with organic matter pools and fluxes underneath *A. longifolia* canopy, compared with the native legume *S. spectabilis*. Furthermore, *A. longifolia* compost was evaluated for the first time as a potential agricultural soil amendment. Degradation of this compost in soil was observed in controlled conditions and its effects on maize growth and kernel quality studied in an urban garden setting.

A. longifolia dune invasion increases the silt-clay fraction, root and rhizosphere biomass. Increased soil phosphorus cycling and lower tissue phosphorus concentrations create an N/P imbalance in the oligotrophic system.

Co-composted *A. longifolia* litter/biomass and biomass compost alone are increasing soil microbial activity. Biomass compost has beneficial effects on maize growth and provides, mixed with nutrient-rich compost, maize productivity levels comparable to mineral fertilization. Further results show open-pollinated maize varieties exhibit increased kernel micronutrient concentrations under compost fertilization, compared to hybrid maize.

Summarized, N/P co-limitation needs to be considered when observing oligotrophic ecosystem invasion. Also, belowground processes precede aboveground effects, making early removal of young plants imperative for ecosystem conservation. Composted biomass can be safely employed as a soil amendment with beneficial effects on various soil and plant parameters, potentially encouraging future eradication.

Keywords

Acacia longifolia, invasive plant, plant-soil interaction, compost, N/P stoichiometry

Table of Contents

Figures and tables	IX
Abbreviations	X
 Chapter 1	
General introduction	
1.1 Facets of the dawning Anthropocene	2
1.1.1 Derailing biogeochemical cycles	
1.1.2 Disrupting biogeographical dispersion of species	
1.1.3 The soil: crucial realm of interaction	
1.2.1 <i>Acacia longifolia</i> : A model plant for invasion	
1.2 <i>Acacia longifolia</i>, a successful invader in oligotrophic systems	5
1.2.2 Oligotrophic dunes: A model system for invasion	
1.2.3 The invaders' impact on the soil: Organic matter pools	
1.2.4 The invaders' impact on the soil: Microbiota and potential nutrient turnover	
1.2.5 The invaders' impact on the soil: Nutrient stoichiometry	
1.3.1 <i>Between a rock and a hard place: Natural ecosystems, agriculture and plant invasion</i>	
1.3 From loose-loose to win-win?	9
1.3.2 Potential agricultural usage of invasive biomass	
1.3.3 <i>Acacia longifolia</i> green waste compost	
1.4.1 General overview	
1.4 General outline and objectives per chapter	12
1.4.2 Chapter 2: <i>How to outgrow your native neighbour?</i>	
1.4.3 Chapter 3: <i>N/P imbalance as a key driver for the invasion?</i>	
1.4.4 Chapter 4: <i>Following the degradation of invasive species green waste compost in a sandy soil</i>	
1.4.5 Chapter 5: <i>Sustainable urban agriculture using compost and an open-pollinated maize variety</i>	
1.5 References	19

Chapter 2

How to outgrow your native neighbour? Belowground changes under native shrubs at an early stage of invasion

2.1 Abstract	25
2.2 Introduction	26
2.3.1 <i>Study site and sampling design</i>	
2.3.2 <i>Soil fractionation and phosphorus determination</i>	
2.3 Materials and Methods	29
2.3.3 <i>Elemental analysis and isotopic ratios</i>	
2.3.4 <i>Statistical analysis</i>	
2.4 Results	31
2.4.1 <i>Invasion pattern and aboveground plant characteristics</i>	
2.4.2 <i>Belowground mass, organic matter and P pools in invaded and non-invaded <i>C. album</i></i>	
2.4.3 <i>Nutrient concentrations and isotopic signatures of < 63 µm soil fraction</i>	
2.4.4 <i>Multiple factor analysis and RV coefficients</i>	
2.5 Discussion	36
2.6 References	40

Chapter 3

N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume

3.1 Abstract	43
3.2 Introduction	44
3.3.1 <i>Study site and species description</i>	
3.3.2 <i>Plant sampling design and in situ measurements</i>	
3.3.3 <i>Soil collection and preparation</i>	
3.3 Materials and Methods	47
3.3.4 <i>Colorimetric assays</i>	
3.3.5 <i>Fluorometric enzyme activity profiling</i>	
3.3.6 <i>Elemental analysis and isotopic ratios</i>	
3.3.7 <i>Statistical analysis</i>	

3.4 Results	51
3.4.1 <i>Pool sizes and stoichiometry</i>	
3.4.2 <i>Specific enzyme activities to assess potential flux rates</i>	
3.4.3 <i>Spatial impact</i>	
3.4.4 <i>Partial least-squares regression</i>	
3.5 Discussion	57
3.6 References	61

Chapter 4

Following the degradation of invasive species green waste compost in a sandy soil using novel high throughput microplate methods for enzyme kinetics and pH: Indications for N immobilization, increased soil quality and a crucial role of available P

4.1 Abstract	65
4.2 Introduction	66
4.3.1 <i>Soil description, compost production and treatment description</i>	
4.3.2 <i>Sampling regime and preparation</i>	
4.3 Materials and Methods	69
4.3.3 <i>Colorimetric nutrient analyses of the soil solution</i>	
4.3.4 <i>pH analysis</i>	
4.3.5 <i>Fluorometric analyses</i>	
4.3.6 <i>Statistical analysis</i>	
4.4 Results	72
4.4.1 <i>Soil solution changes along time</i>	
4.4.2 <i>Changes in V_{max} and K_m</i>	
4.4.3 <i>Net nitrification rates and correlations</i>	
4.5 Discussion	76
4.6 References	79

Chapter 5

Sustainable urban agriculture using compost and an open-pollinated maize variety

5.1 Abstract	83
5.2 Introduction	84
5.3.1 <i>Sample dates, setup and preparation</i>	
5.3 Materials and methods	87
5.3.2 <i>Plant variety and phenology</i>	
5.3.3 <i>Soil collection, preparation and organic matter analysis</i>	
5.3.4 <i>Ionomics, total carbon, nitrogen and stable isotope analysis</i>	
5.3.5 <i>Statistical analysis</i>	
5.4.1 <i>Soil changes</i>	
5.4 Results and discussion	90
5.4.2 <i>Effects on plant biomass and nitrogen</i>	
5.4.3 <i>Grain micronutrient concentrations in commercial and open- pollinated varieties</i>	
5.4.4 <i>Conclusions</i>	
5.5 References	98

Chapter 6

General Discussion

6.1 Rhizosphere-litter-SOM interactions during the invasion process	102
6.1.1 <i>Litter decomposition and its effects on SOM buildup</i>	
6.1.2 <i>Rhizosphere: Contributing the nutrients for SOM degradation?</i>	
6.1.3 <i>Belowground OM dynamics and the impact on N and P of the surrounding vegetation</i>	
6.2 Composting <i>A. longifolia</i> biomass: Mimicry of its soil impacts?	107
6.2.1 <i>From natural degradation to green waste compost</i>	
6.2.2 <i>Living up to the reality test? An urban case study of <i>Acacia longifolia</i> green waste compost</i>	
6.3 Conclusion	111
6.4 References	112

Figures and tables

Figure 1.1 <i>A. longifolia</i> native and invasive range.	5
Figure 1.2 <i>A. longifolia</i> invasion in the dune system, known effects.	6
Figure 1.3 Potential benefits of <i>Acacia spp</i> removal from agricultural systems with adjacent native ecosystems.	9
Figure 1.4 Illustrated summary of chapter 2.	13
Figure 1.5 Illustrated summary of chapter 3.	14
Figure 1.6 Illustrated summary of chapter 4.	16
Figure 1.7: Illustrated summary of chapter 5.	17
Table 2.1 Differences between growth form and foliar nutrient content as well as stable isotope values of <i>Acacia longifolia</i> , invaded (+ Inv) and non-invaded <i>Corema album</i> (- Inv).	31
Figure 2.1 Kernel density estimation of <i>Corema album</i> size, expressed in volume (m ³).	31
Figure 2.2 Belowground mass pools underneath non-invaded (- Inv) and invaded (+ Inv) <i>Corema album</i> plants.	32
Figure 2.3 Effects of plant-derived OM and P on foliar values and the mass ratio differences in <i>C. album</i> , invaded and non-invaded by <i>A. longifolia</i>	33
Table 2.2 Nutrient concentrations and isotopic signatures of soil fractions < 63 µm of invaded (+ Inv) and non-invaded <i>Corema album</i> (- Inv).	34
Figure 2.4 First two dimensions of a multiple factor analysis on parts of the data set from Figure 2.2 , Table 2.1 and Table 2.2 .	35
Table 2.3 RV coefficients of matrices used in Figure 2.4 , as well as a matrix of mass ratios (plant contribution to total OM and P total) as shown in Figure 2.3 .	36
Figure 3.1 Schematic of the experimental setup.	47
Table 3.1 Characterization of the biomass pools associated with the invasive <i>Acacia longifolia</i> and the native <i>Stauracanthus spectabilis</i> legumes.	51
Table 3.2 Characteristics of the soil and the rhizosphere compartments of the two studied legumes, the invasive <i>Acacia longifolia</i> and the native <i>Stauracanthus spectabilis</i> .	52
Table 3.3: Specific extracellular enzyme activities of rhizosphere and litter pools, related to organic matter content.	53
Figure 3.2 Distance effects of invasive and native legumes to <i>C. album</i> foliar N and δ ¹⁵ N.	54
Figure 3.3 Effect of legume presence on <i>C. album</i> foliar N and δ ¹⁵ N.	55
Figure 3.4 Relationship between change in <i>C. album</i> foliar total N and P.	55
Figure 3.5 Biplots showing sample scores (dots) and loadings of selected variables (labelled arrows) on the first two components of partial least squares regression (PLSR) models.	56
Figure 4.1 Changes in nitrate and ammonium concentrations along time	72
Figure 4.2 Changes in phosphate concentrations and N:P ratios in the soil extract along time.	73
Figure 4.3 Changes in pH and maximum rate of activity (V _{max}) of various enzymes.	74
Figure 4.5 Correlations of NH ₄ ⁺ - N nitrified to NO ₃ ⁻ at day 174 with PO ₄ ³⁻ concentration and apparent K _m of Nag = N-acetylglucosaminidase	75
Figure 4.4 Amount of NH ₄ ⁺ - N nitrified to NO ₃ ⁻ after adding 50 mg NH ₄ ⁺ per kg soil.	75
Table 5.1 Topsoil parameters (< 20 cm) of untreated soil (control), soil with municipal compost (MC) and soil with a mix of green waste compost and municipal compost (GWC/MC).	90
Figure 5.1 Differences in plant and grain weight of both corn varieties in all treatments.	91
Table 5.2 Nutrient concentrations in maize grain of plants growing in soil without treatment (Control), soil with municipal compost (MC) and soil with a compost-mix (GWC/MC) of green waste compost (GWC) and MC.	92
Figure 5.2 Pearson correlation between grain nitrogen (N) content and soil organic matter (OM).	93
Figure 5.3 Nutrient Network between all macro- and micronutrients found in the maize kernels.	94
Figure 5.4 RV-coefficient matrix of data matrices used in this work.	95
Figure 5.5 Multiple factor analysis (MFA) of variables related with the maize plants (n = 13 per plant variety).	96
Figure 6.1 <i>A. longifolia</i> invasion in the dune system, known effects plus additional effects found here.	104

Abbreviations

- **Inf** = Plant growing far away (> 5 m) from any *A. longifolia* plant

+ **Inf** = Plant with an *A. longifolia* plant growing inside its canopy

$\delta = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] * 1000$, where R is the ratio between the heavier and lighter isotopes

ΣC = β-glucosidase + β-xylosidase + β-glucuronidase + cellobiohydrolase

AMF = Arbuscular mycorrhizal fungi

AOB = Ammonium oxidizing bacteria

Ap = Acid phosphatase

C = Carbon

Cel = Cellobiohydrolase

CL = *A. longifolia* biomass mixed with chipped *A. longifolia* litter in a 1:10 ratio

CO = *A. longifolia* biomass compost

EA = Enzymatic activities

ECM = Ectomycorrhiza

EEA = Extracellular enzyme activities

GHG = Greenhouse gas

Gla = β-glucosaminidase

Glc = β-1,4-glucosidase

Glu = β-glucuronidase

GWC = Green waste compost

IB = Incubation buffer

IN = *C. album* plants growing in *A. longifolia* canopy

K_m = Measure of enzyme substrate affinity

MC = Municipal compost

MFA = Multiple factor analysis

MIC = microbial biomass

MU = 4-Methylumbelliferyl

N = Nitrogen

Nag = β-1,4-N-acetylglucosaminidase

N_{inorg} = NO₃⁻ and NH₄⁺

Nit = Nitrification

N_{tot} = Total nitrogen

OM = Organic matter

OPV = Open pollinated variety

OUT = *C. album* plants growing outside of *A. longifolia* breakpoint range

P = Phosphorus

PLSR = Partial least squares regression

P_{org} = Organic phosphorus

P_{tot} = Total phosphorus

r² = Person's correlation

RES = Basal respiration

r_s = Spearman's rank correlation

RS = Rhizosphere

SOC = Soil organic carbon

SOM = Soil organic matter

SON = soil organic nitrogen

UI = Yamada universal indicator dye

VIP = Variable importance in the projection

V_{max} = Maximum reaction rate of an enzyme

Xyl = β-xylosidase

Chapter 1

General introduction

1.1 Facets of the dawning Anthropocene

Many scientists argue that since the beginning of the industrial revolution, a new era for the planet has dawned, the Anthropocene. This era is marked by human activity inducing fundamental changes of the earth's biogeochemical cycles (Crutzen, 2002) and the biogeographical dispersion patterns of species (Capinha *et al.*, 2015). These alterations are pushing several of the earth's systems to their boundaries, potentially leading to abrupt changes that could be highly detrimental for ecosystems and thus human survival on a global scale (Rockström *et al.*, 2009).

1.1.1 Derailing biogeochemical cycles

Among the most impacted biogeochemical cycles are the nitrogen (N) (De Vries *et al.*, 2013) and the phosphorus (P) (Carpenter and Bennett, 2011) cycles. However, the human impact that is exercised on these nutrient cycles is in no way straightforward. While in some regions human inputs of N and/or P are leading to degradation of natural ecosystems via eutrophication, other ecosystems are lacking either one or both of these essential nutrients, therefore being unable to sustain agricultural activities necessary for local populations (Carpenter and Bennett, 2011). This situation increases the human dilemma of N and P cycling, because the main response to population increase is the intensification of agricultural production, fuelled by an increment in mineral fertilizer input. Based on recent estimates (Conijn *et al.*, 2018), this situation is quite dramatic, as the planetary boundaries for these nutrient cycles have potentially already been transgressed in 2010 and will further deteriorate

drastically without urgent action. Lastly, both cycles are also connected to the C cycle, for example through greenhouse gas (GHG) emissions related to agricultural activities and the industrial fixation of N, thus connecting biosphere integrity to other crucial planetary boundaries, like climate change (Steffen *et al.*, 2015).

1.1.2 Disrupting biogeographical dispersion of species

The transport of species between continents by humans, wittingly as well as unwittingly, has led to a great dispersal of species with an initially local range over the whole globe, leading some authors to term the Anthropocene also as the “Homogenocene” (Zalasiewicz *et al.*, 2018), due to the rapid homogenization of many ecosystems worldwide. At present, human activity has displaced and naturalized around 4 %, of the global vascular flora, which is approximately equivalent to the size of the European flora (Van Kleunen, *et al.*, 2015). When these naturalized plants persist in an novel ecosystem, they might simply coexist with the native fauna and flora. In some cases, however, these exotic species can turn invasive, thus reproducing without further human influence, subsequently spreading from their introduction sites and further dispersing into native surroundings (Richardson *et al.*, 2000). While only a small percentage of plants turn invasive, they are at the basis of the food web and were therefore found to be highly represented as invasive species with major impact (Mollot *et al.*, 2017). Invasive plant species can be highly detrimental for the functioning

of ecosystems (Vilà *et al.*, 2011) and they can act as “ecosystem engineers”, fundamentally changing the entire system (Crooks, 2002). Strong ecosystem engineers are especially found within the subgroup of invasive woody plants, shrubs and trees, which comprise 21 species on the 100 world’s worst invasive species list (Lowe *et al.*, 2000). The term “ecosystem engineer” is used for organisms that physically change ecosystems as to modulate the resource availability for themselves as well as other species. In the context of trees, they do so using their own biomass, thus they are so-called autogenic ecosystem engineers (Jones *et al.*, 1994). These autogenic engineers might change soil surface layers, thus excluding other species or accumulate topsoil and increase organic matter (OM) and nutrient availability, thus benefitting other species. While these are effects mediated by aboveground biomass, such as litter, their belowground effects can be as pervasive, for example by creating a root layer that changes nutrient availability, stabilizes the soil and binds sediments (Fei *et al.*, 2014). These biogeomorphic impacts are a point of interaction, where biogeochemical and biogeographical effects of human disturbance meet at soil level, with profound and potentially irreversible impacts for the ecosystem in question.

1.1.3 The soil: crucial realm of interaction

When looking at the planetary boundaries proposed for a safe human operating space (Rockström *et al.*, 2009), it becomes clear that in order to understand their real limits, it is crucial to decipher the interaction between them (Mace *et al.*, 2014). The soil is the most important interaction point between biogeochemical and biogeographical effects. Its capacity to sustain plant growth and maintain nutrient cycling is based on the functioning of its biotic compartment, also termed the “soil health” (Doran and Zeiss, 2000). For invasion science, the soil, or rather the soil biota, have become an increasing focal point, which is in need of significant research attention (Ricciardi *et al.*, 2017). It is known that plant-soil interactions and their effects on nutrient cycling are important to understand the impact of invasive plants, especially for N and C cycling (Ehrenfeld, 2003). This was found to be particularly the case for woody and N-fixing plants, with effects including increased soil N-cycling and net primary production (Liao *et al.*, 2008). However, the simultaneous increase in N-cycling and biomass production by invasive plants has been described as a paradox (Rout and Callaway, 2009), as invasive plants often create monospecific vegetation patches, thus decreasing potential niche partitioning for nutrients, which should also decrease primary production and nutrient retention (Hooper *et al.*, 2005). A possible explanation for this inverse diversity–productivity relationship could be positive plant-soil interactions exhibited by invasive plants, which benefit from the soil biota encountered in the novel environments, thus sustaining

their monocultural growth (Kulmatiski *et al.*, 2012). This framework of plant-soil interactions and its relationship with nutrient cycling and productivity connects natural sciences, such as invasion science, with agricultural sciences (Mariotte *et al.*, 2018) and the cycles of some of the most important plant nutrients: N and P. Indeed, while quite some information is available about the effects of invasive plants on the N-cycle, the information about interaction between N and P cycle is still sparse (Hu *et al.*, 2019). However, they need to be observed together, as for example increased N input might lead to a deficit in P (Penuelas *et al.*, 2013) and both the N and the P cycle are strongly connected at soil level with each other as well as with the C cycle (Cleveland and Liptzin, 2007). This is due to the requirements of the heterotrophic soil microbial community, which produces extracellular enzymes to meet their C demand for energy, while scavenging for P and N (Sinsabaugh *et al.*, 2009), ultimately driving plant growth. Thus, the observation of the whole plant-soil system and the nutrient exchanges within and between them are of crucial importance to understand the complex issues connected to the Anthropocene, such as plant invasions and changes in biochemical nutrient cycling (deB Richter *et al.*, 2015).

1.2 *Acacia longifolia*, a successful invader in oligotrophic systems

1.2.1 *Acacia longifolia*: A model plant for invasion

Among the invasive woody plants, the genus *Acacia* dominates many areas worldwide (Richardson and Rejmánek, 2011). Within this genus especially the Australian acacias are highly invasive, with the highest invasion rates (22 of 1020 species are invasive) for any large lineage within the legume family (Miller *et al.*, 2011). As circa one third of the world's land surface is suitable for the growth of Australian acacias and many species are commercially important, they make for an excellent model group to study the impact of plant invasions on a global scale (Richardson *et al.*, 2011). One of the species within the Australian acacias that is considered “very widespread” globally is *Acacia longifolia* (Richardson and Rejmánek, 2011), with particular relevance in the Mediterranean climate areas. In Europe (figure 1.1, middle), it has spread specifically in southern European coastal dunes

(Marchante *et al.*, 2015). In Portugal (figure 1.1, right), this species was deliberately introduced for dune stabilization at the beginning of the 20th century and subsequently turned invasive (Marchante *et al.*, 2003), with highly detrimental impact for native biodiversity. As it exhibits a larger size and higher growth rates than the native vegetation, it alters the vegetation structure (Rascher *et al.*, 2011) and acts as an ecosystem engineer (Hellmann *et al.*, 2011). Apart from physiological traits related to biomass production, *A. longifolia* also exhibits increased resistance to salinity (Morais *et al.*, 2012), a large seed bank (Marchante *et al.*, 2010) and an overall high phenotypic plasticity (Peperkorn *et al.*, 2005; Máguas *et al.*, 2011). These traits make its eradication difficult, as even if the aboveground part of the plants are removed, their legacy effects on the soil remain, turning the restoration of the original ecosystem almost impossible (Marchante *et al.*, 2011)

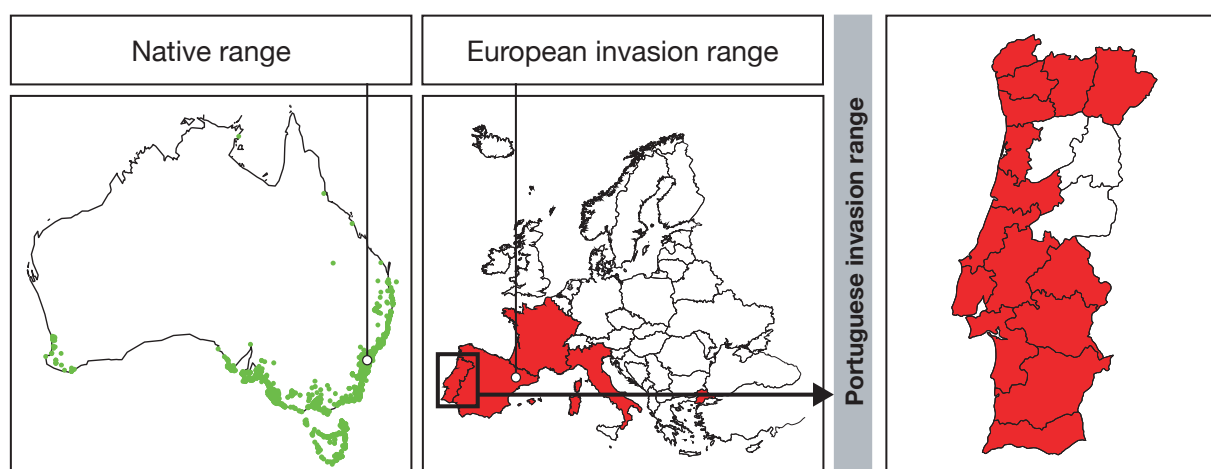


Figure 1.1 *A. longifolia* native and invasive range.

Left: Native range distribution map of *Acacia longifolia* adapted from Klock *et al.*, 2016

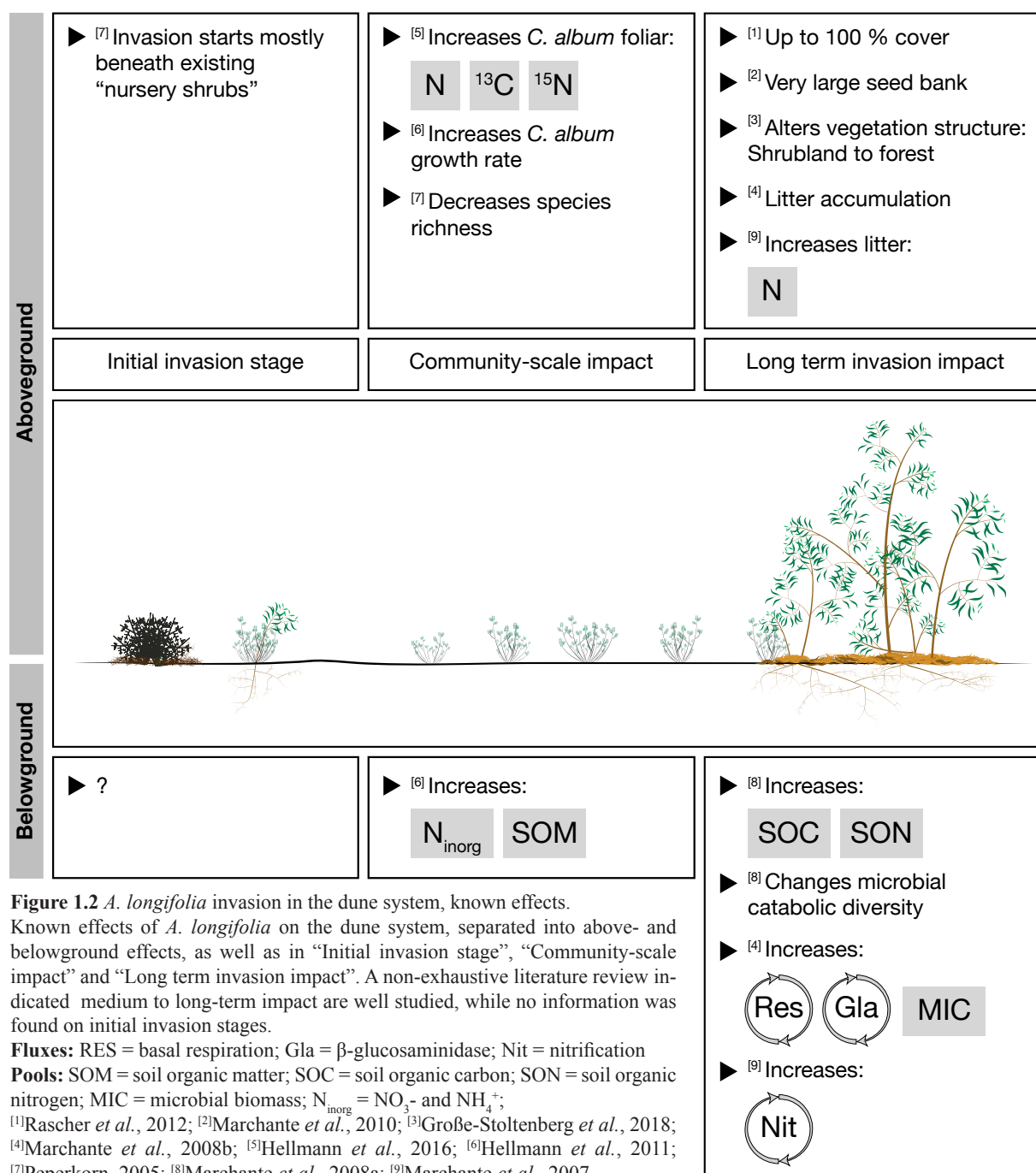
Middle: Distribution of *Acacia longifolia* in its invasive range in Europe adapted from <http://www.europe-aliens.org/default.do>

Right: Distribution of *Acacia longifolia* in its invasive range in Portugal adapted from <http://invasoras.pt/>.

1.2.2 Oligotrophic dunes: A model system for invasion

Acacia longifolia invasion has severe impacts on oligotrophic dunes (**figure 1.2**), which are biodiverse and often unique ecosystems that are essential for the coastal integrity (Marchante *et al.*, 2015). Due to human activities in the last 100 years, only 25 % of the Mediterranean dunes are still in a “natural state”

(Salman and Strating, 1991). Furthermore, as dunes are naturally dynamic, they provide ample opportunity for plant invasions (Doody, 2013), putting the remaining intact dunes at risk. Interestingly, oligotrophic systems were long considered to be less prone to invasion, which is an assumption challenged in the last decades (Funk and Vitousek, 2007). The invasion of oligotrophic ecosystems by *A. longifolia*



lia is probably related to its capacity to fix N and its high resource use efficiency (Werner *et al.*, 2010), which translates into continuous growth and increased ecosystem primary production, thus turning shrubland into forest (Große-Stoltenberg *et al.*, 2018). Even though *A. longifolia* ultimately dominates the ecosystem, there is a period in which its presence is beneficial for the native dune vegetation, as was found for a native shrub (*Corema album*) in the Portuguese dunes (Hellmann *et al.*, 2011). *C. album* exhibits increased foliar N and higher growth rates when growing closer to *A. longifolia*, which is an effect surpassing its canopy (Rascher *et al.*, 2012). However, while various hypothesis were put forward as to the origin of this paradoxically positive effect, such as N input from accumulated litter or mass flow of fixed N to the surrounding vegetation, the mechanism of this N effect and when it starts to occur during invasion are not clear.

1.2.3 The invaders' impact on the soil: Organic matter pools

In order to elucidate the effects of *A. longifolia* on the dunes, belowground changes require special attention, as they often predate aboveground effects (Vila *et al.*, 2011). *A. longifolia* exhibits extensive litter fall, and litter layers continuously thicken the longer the invasion is taking place (Marchante *et al.*, 2008b). Also, root mass is known to increase strongly after *Acacia* invasion (Morris *et al.*, 2011), which both together increase soil organic C in the topmost (ca. 10 cm) soil layer (Marchante *et al.*, 2007). Apart from the general biomass increment on soil level, especially soil organic matter (SOM) in the topsoil (Hellmann *et al.*,

2011), as well as total N and microbial biomass increase in long term invaded areas (Marchante *et al.*, 2008b). While these observations are important to decipher general effects, it is not clear how this OM is distributed in the poor sandy soils of the dunes. For example, SOM might stabilize in certain soil fractions (e.g. the silt-clay fraction) more than in others (Six *et al.*, 2002), which is a process occurring in other oligotrophic systems upon plant invasion (Schwarz *et al.*, 2016). Also, the rhizosphere, an important factor to understand plant invasions (Philippot *et al.*, 2013), is likely to be a compartment exhibiting increased SOM levels, as it is in direct contact with the roots and thus more rapidly impacted by the invasive plant.

1.2.4 The invaders' impact on the soil: Microbiota and potential nutrient turnover

Not only the impacts of *A. longifolia* on soil C and N pools, but also on the cycling of C and N in the invaded dunes have been extensively studied and are profound (Marchante *et al.*, 2007, 2008a, 2008b, 2009). Putative reasons for these impacts could be the capacity of *A. longifolia* to create various types of symbiotic relationships with microorganisms related to N acquisition and nutrient uptake in general. They form arbuscular mycorrhiza (Rodríguez-Echeverría *et al.*, 2009), as well as ectomycorrhizal symbiosis (Martin *et al.*, 2002) and nodulation with both *Rhizobium* and *Bradyrhizobium* species (Rodríguez-Echeverría *et al.*, 2007). This has lead some authors to propose an “invasional meltdown” of *A. longifolia*, which describes the co-introduction of symbiotic microorganisms from its home range (Rodríguez-

Echeverría, 2010). However, while there are indications of homogenization and adaptation of the soil microbial community to the presence of *A. longifolia*, its initial establishment also requires effective nodulation with the microorganisms already present (Le Roux *et al.*, 2018). In any case, many effects related to *A. longifolia* invasion are connected to changes in nutrient fluxes such as increased β -glucosaminidase activity (Marchante *et al.*, 2004; 2008b; 2009), basal respiration (2008b) as well as potential nitrification (Marchante *et al.*, 2007; 2008b) and changes in catabolic diversity (Marchante *et al.*, 2008a). Thus, apart from the microbial diversity itself, an important tool to quantify and understand soil nutrient cycling is the analysis of extracellular enzyme activities (EEAs), which are mediating the degradation of complex organic matter into assimilable subunits such as amino acids, anorganic ions or sugars (Caldwell, 2005). Therefore, using EEA profiles can provide crucial data about the functional adaptation of microorganisms in the rhizosphere and on the root surface (Pritsch *et al.*, 2009). Of the many possible extracellular enzymes that can be measured, the most used are β -1,4-glucosidase (Glc), β -1,4-N-acetylglucosaminidase (Nag) and acid phosphatase (Ap) (Sinsabaugh *et al.*, 2009), as they can function as proxies for C, N and P turnover, respectively.

1.2.5 The invaders' impact on the soil: Nutrient stoichiometry

The flux rates between and within biomass pools, as well as the soil plant interaction of nutrient exchange is heavily reliant on the stoichiometric relationship between N, C and P (Manzoni *et al.*, 2010). However, most of the

work on soil changes after *A. longifolia* invasion is mainly concerned with the C and the N cycles, while the P cycle has been largely neglected. Yet, it is very important to understand the former two, as all three cycles are tightly connected on soil level through the processes of microbial degradation (Sinsabaugh *et al.*, 2008). Especially the relationship of the N and the P cycle in plant invasion requires further attention (Sardans and Peñuelas, 2012) and as a prolific N fixer, it is clear that *A. longifolia* will interfere with existing N and P relationships in its novel environments. Of special importance to understand the cycling and ultimately uptake of N and P by plants is the rhizosphere (Richardson *et al.*, 2009). For *Acacia spp.*, it was recently found that plant growth promoting rhizobacteria are overrepresented in this compartment (Kamutando *et al.*, 2019) and they seem to be especially important for N and P cycling (Kamutando *et al.*, 2017). Furthermore, as was found in a recent meta-analysis, rhizosphere and litter differentially affect soil biota in plant invasions, which urges further research into these compartments (Zhang *et al.*, 2019). In summary, as Hulme *et al.*, (2013) state, it is crucial to look at pools, fluxes and nutrient stoichiometry at the same time to understand plant invasions. In the case of an ecosystem engineer like *A. longifolia* in an oligotrophic ecosystem, SOM, roots and litter mass are the main belowground pools that are changing and their fluxes will be determined by N/P stoichiometries and the potential turnover rates as measured using tools like EEA analysis.

1.3 From loose-loose to win-win?

1.3.1 Between a rock and a hard place: Natural ecosystems, agriculture and plant invasion

Plant invasions are not arbitrary in their spatial distribution, but rather connected to anthropogenic disturbances and regions of human activities, such as urban and agricultural areas (Chytrý *et al.*, 2009). The Mediterranean Basin has seen millennia of human interference and it is likely that especially woody plants will continue to be very problematic invaders in this area (Fox, 2012). Furthermore, the agricultural intensification in the Mediterranean

region occurring currently is projected to increase plant invasions (Chytrý *et al.*, 2012), thus increasing pressure on the adjacent native vegetation. At the same time, destruction of soil function through intensive farming techniques, such as deep tillage and the abundant use of mineral fertilizers leads to losses of topsoil, therefore increasing nutrient leaching into the surrounding vegetation (Zalidis *et al.*, 2002). The resulting overabundance of nutrients degrades nutrient-poor ecosystems like the Mediterranean directly, for example by disconnecting existing plant-soil relationships

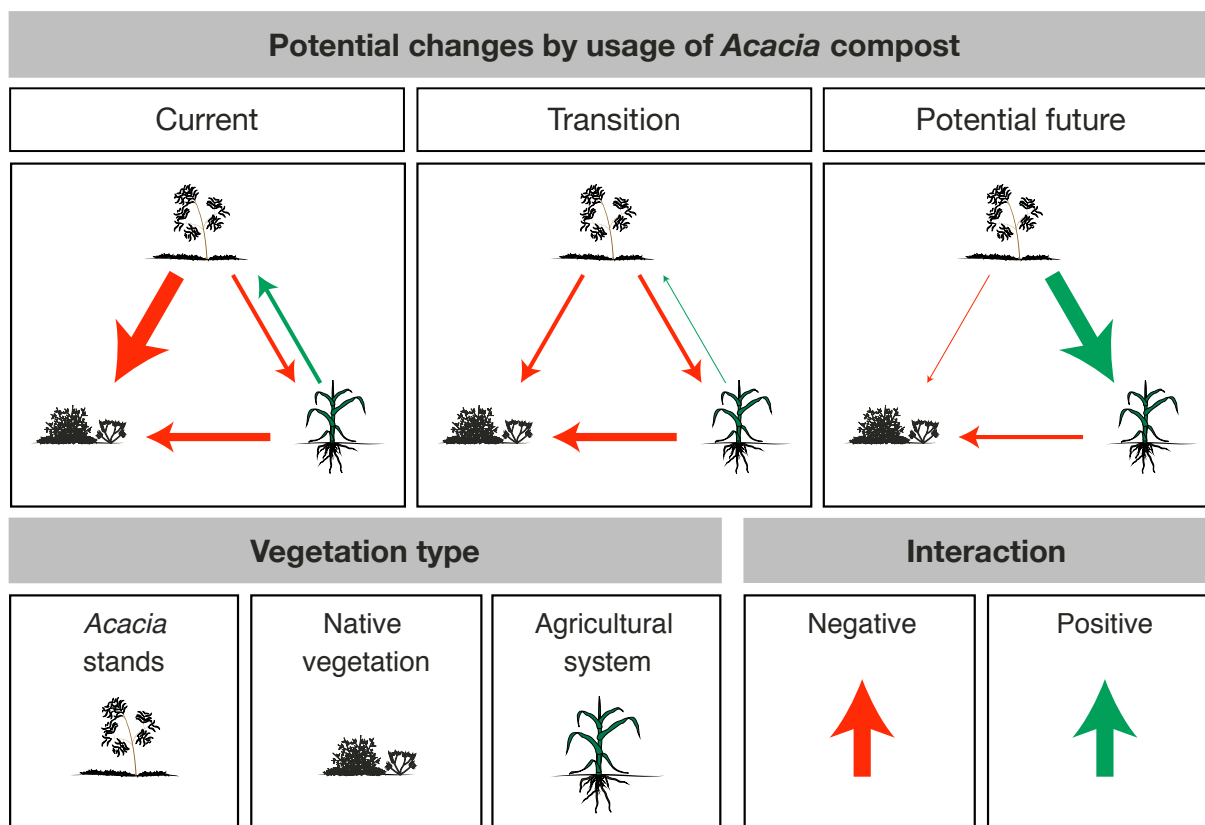


Figure 1.3 Potential benefits of *Acacia* spp removal from agricultural systems with adjacent native ecosystems. In the “current” state, the invasive species have negative impacts on the agricultural systems as well as on the native ecosystems by competing for nutrients and space. Also, nutrient leaching from agricultural systems has negative impacts on native ecosystems. With the removal of *Acacia* stands, native ecosystems can regrow and the negative impacts of the invasive decreases (“Transition”). As the invasive biomass is consequently harvested, negative effects of the invasive plants further decrease and if that biomass is then used as soil amendment to increase agricultural soil quality, the negative effects will turn positive, as fertilizer and water input can be decreased (“Potential future”).

through alleviating N-limitation (Dias *et al.*, 2017), or in other ecosystems, also by increasing levels of available P (Wassen *et al.*, 2005). Native ecosystems are therefore under double pressure from both the invasion of invasive species as well as from leached nutrients of adjacent agricultural plots. Additionally, excess nutrients might increase plant invasion, as they can often benefit the most from high resource availability (Catford *et al.*, 2012). The current situation thus constitutes a loose-loose-loose scenario (**Figure 1.3**) in which native ecosystems loose as they suffer negative impacts from both agriculture and invasive species, while for the farmers, invasive species are a nuisance, competing with their crops for space and requiring control, ultimately creating an economic loss. Lastly, agricultural practices disturb adjacent ecosystems and degrade soil health, increasing nutrient leaching and nurturing the invasive plants.

1.3.2 Potential agricultural usage of invasive biomass

While the long term impacts of plant invasions on native ecosystems are clearly negative, they might have more nuanced and differentiated effects on human livelihoods (Shackleton *et al.*, 2019). For example, in the case of invasive *Acacia*, negative impacts can be loss of farmland and diminishing crop growth, while its usage as firewood or for fencing, as well as the shade it provides are regarded as positive (Ngorima *et al.*, 2019). As *Acacia spp.* are highly problematic invaders in Portugal, interest in using them for various purposes is growing, mainly in order to alleviate costs related to their continuous management (Souza-Alonso *et al.*, 2017). Locally, *Acacia* can be

used for perfume production or food, however due to the minimal silvicultural input, they are especially well suited for C and energy storage, for example as fuelwood (Griffin *et al.*, 2011). Indeed it was shown that the usage of biomass for energy from eradication measures can to potentially minimize eradication costs in Portugal (Carneiro *et al.*, 2014). While this is potentially useful for off-grid energy demands (Griffin *et al.*, 2011) there might be also a more immediate use for *Acacia* biomass for agricultural purposes. One recent approach is using *A. longifolia* residues directly as green manure in order to fertilize crops and decrease weed emergence (Souza-Alonso *et al.*, 2018). However, this application, if not well employed, could lead to a further spread of the species through the inadvertent introduction of viable seeds from the residues.

1.3.3 *Acacia longifolia* green waste compost

A potential way to degrade viable seeds and diminish phytotoxicity before applying *A. longifolia* biomass as a soil amendment is to use its shredded biomass as a feedstock for green waste compost (GWC) (Brito *et al.*, 2013). The resulting GWC has been shown to be stable and deemed useful both as a soil amendment as well as a horticultural substrate if mixed with other feedstock, such as pine bark (Brito *et al.* 2015a). In subsequent tests using pure *Acacia* compost, produced from a mix of *A. longifolia* and *A. melanoxylon* residue feedstock, lettuce was found to grow similarly well than on commercial pine bark compost substrate (Brito *et al.*, 2015b). A field scale test of *Acacia* compost as a soil amendment, however, has not been performed

yet and is needed to inform local stakeholders, if it is safe to use and has the benefits of other GWCs used as soil amendments. In case the application of GWC were successful in an agricultural setting, the loose-loose-loose situation described might turn gradually into a situation where *Acacia* harvest and production of GWC leads to decreased pressure on the native system and less nutrient leaching (**Figure 1.3**, “Transition”). At a final stage, continuous harvesting of *Acacia* would lead to a beneficial situation, where soil quality increase through GWC amendments decreases nutrient leaching up to the point where it has a negligible effect on surrounding vegetation, while natives can reclaim the areas previously occupied by *Acacia* (**Figure 1.3**, “Potential future”). Indeed, the usage of GWC for agricultural purposes is increasingly recognized as an important tool to close biomass cycles and create a valuable organic soil amendment (Reyes-Torres *et al.*, 2018). This was found to be especially the case close to peri-urban farm land (Eldridge *et al.*, 2018), which is also an area under heavy pressure from invasive plants (Chytrý *et al.*, 2009). Thus, these areas could be good starting points for the application of GWC derived from *A. longifolia* eradication measures. Apart from the usage of this compost per se, however, there should also be a consideration as to the agricultural system used with this compost.

While SOM increase induced by compost addition is beneficial for crop yields in intensive agricultural systems (Scotti *et al.*, 2016), increased soil health might be even more important in organic farming systems, making, as an end result, also for healthy food (Reeve *et al.*, 2016). In summary, while employing *A. longifolia* GWC in an agricultural setting has a good chance of being beneficial, its usage should first be studied in a holistic manner, including plant and soil health as well as nutritional quality of the crop produced.

1.4 General outline and objectives per chapter

1.4.1 General overview

The work presented here focuses on the effects of the invasive legume *A. longifolia* on soil nutrient cycling and soil organic matter pools. It is mainly concerned with the degradation of its biomass in the soil and the changes implicated with this degradation on the N and P cycles, both in natural ecosystems as well as in agricultural settings. The following chapters can be divided into two parts: Part one consists of chapters 2 and 3, which are concerned with the effects of *A. longifolia* on the surrounding native vegetation, taking advantage of a known system of plant-plant interaction to elucidate invasion impact on soil level. Part two is comprised of chapters 4 and 5, which describe the effect of *A. longifolia* GWC as a soil amendment for agriculture, starting from its degradation and its impact on the nutrient flux in a sandy soil under N fertilization (chapter 4) to its usage and effect on maize (*Zea mays*) crops grown in an organic agricultural setting. The system of plant-plant interaction used for chapters 2 and 3 makes use of the effect of *A. longifolia* on nearby *C. album* plants, which is a native plant that grows abundantly in the Portuguese dunes. It is a shrub belonging to the Ericaceae family, so it is not capable of N fixation itself and as soil organic matter and nutrient levels are naturally extremely low in the dune systems, the only major sources of N are leguminous dune species capable of fixing atmospheric N₂, such as the invasive tree *A. longifolia* or the sclerophyllous native shrub *Stauracanthus spectabilis* (Hellmann *et al.*, 2011). As legumes exhibit less depleted tissue $\delta^{15}\text{N}$ values due to their atmospheric N₂

fixation, foliar $\delta^{15}\text{N}$ can be used as a tracer for plant soil interaction (Unkovich, 2013). This makes *C. album* a good indicator plant for the effect of *A. longifolia* on the N cycle in these oligotrophic systems, as it exhibits naturally very depleted foliar $\delta^{15}\text{N}$ values and is very abundant in the dunes (Rascher *et al.*, 2011).

As this system was already successfully employed to describe of plant-plant interactions (Hellmann *et al.*, 2011; 2016; Rascher *et al.*, 2011), chapters 2 and 3 make use of *C. album* foliar $\delta^{15}\text{N}$ signatures to gain insights into plant-soil interactions. While SOM matter pools were measured in both chapters, chapter 3 adds a dimension of potential turnover of this organic matter by assessing EEAs of various compartments. The EEA measurements were also performed in chapter 4, in order to decipher the impacts of *A. longifolia* GWC on the N and P dynamics in a sandy agricultural soil. Finally, chapter 5 explores the usage of this GWC on maize yield, expanding the nutrients measured from N and P to an ionic analysis, spanning various micro- and macro-nutrients as well as heavy metals.

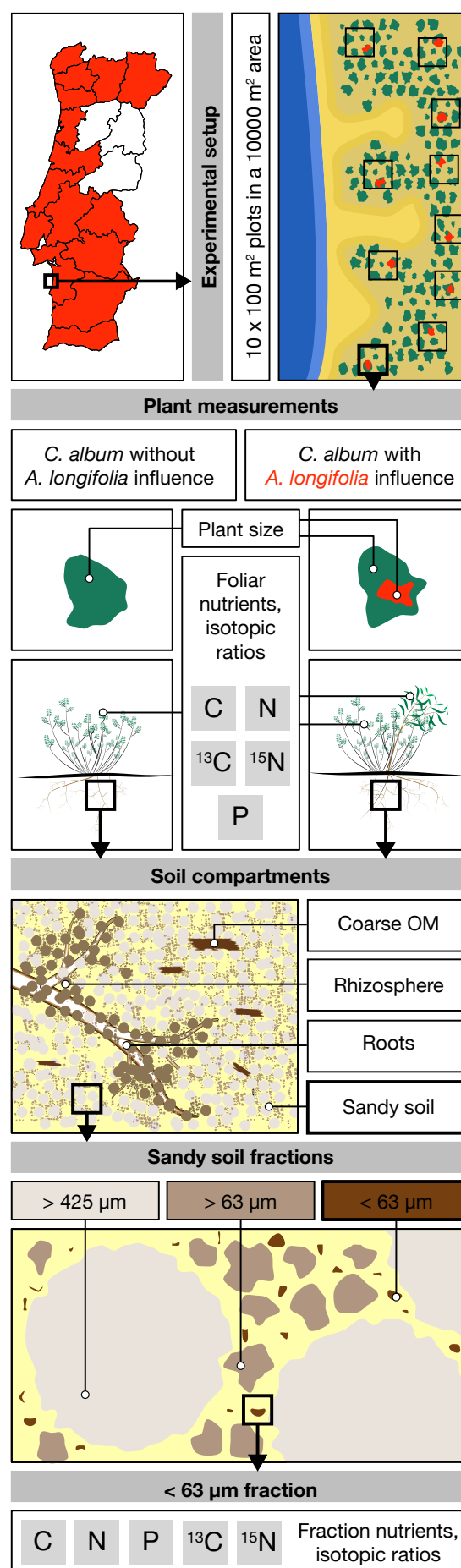
Using this approach, this work describes some fundamental aspects of the N/P imbalance induced by the addition of biomass from an invasive woody legume, while also giving a potential future use to this biomass as a soil amendment in an agricultural setting.

1.4.2 Chapter 2: How to outgrow your native neighbour?

In this chapter a known plant-plant interaction model was used to understand SOM changes at the initial stages of the invasion. As the exact pathway of N distribution into the surrounding vegetation is unknown, an experimental setup was created that excludes aboveground interaction by choosing *A. longifolia* plants at a growth stage that did not allow yet for the formation of a litter layer (**Figure 1.4**). Using this situation, the hypothesis of N distribution solely via belowground pathways could be explored. In a first step foliar changes of *C. album* known to occur in later stages of invasion (increased N, less depleted $\delta^{15}\text{N}$ signatures) were assessed to show that *A. longifolia* indeed has an effect even in early invasion stages, where the litter layer has not yet developed and the autogenic engineering effects are negligible. Then, soil samples were taken and the changes belowground were analysed in order to find the compartments related with the foliar changes observed. Apart from the main OM compartments, soil fractions of the sandy soil were observed, hypothesising changes in the silt-clay fraction as the main fraction for SOM stabilisation.

Figure 1.4 Illustrated summary of chapter 2.

Depicted are the localisation of the sampling site (Pinheiro da Cruz, Portugal), the experimental setup in the primary dunes, as well as the analysis the samples were subjected to. Plots were laid out as 10 x 10 m squares containing various *C. album* plants and at least one *A. longifolia* individual growing inside a *C. album* plant. Plant measurements taken in the field were height and canopy size, estimated from two diameters. After these measurements, foliar samples were taken and analysed for C, N and P, as well as stable isotopic ratios of C and N. At the same site, soil samples were taken and separated into the compartments: Coarse OM, rhizosphere, roots and sandy soil. The sandy soil was then further divided by sieving into 3 fractions: $> 425\ \mu\text{m}$, $> 63\ \mu\text{m}$, $< 63\ \mu\text{m}$. As the smallest fraction ($< 63\ \mu\text{m}$) was found to have high OM levels, it was further analysed for C, N and P, as well as stable isotopic ratios of C and N.



1.4.3 Chapter 3: N/P imbalance as a key driver for the invasion?

This chapter explores the situation of a later stage invasion by *A. longifolia* and uses the same model system with *C. album* foliar N and $\delta^{15}\text{N}$ values as described for chapter 2 in order to test differences between native and

invasive legumes to find differences explaining the invasives species success. In this case, a comparative approach was used, with *A. longifolia* as the invasive legume and *Stauracanthus spectabilis* as a native legume in order to test which compartments underneath the legumes correspond to the impact they have

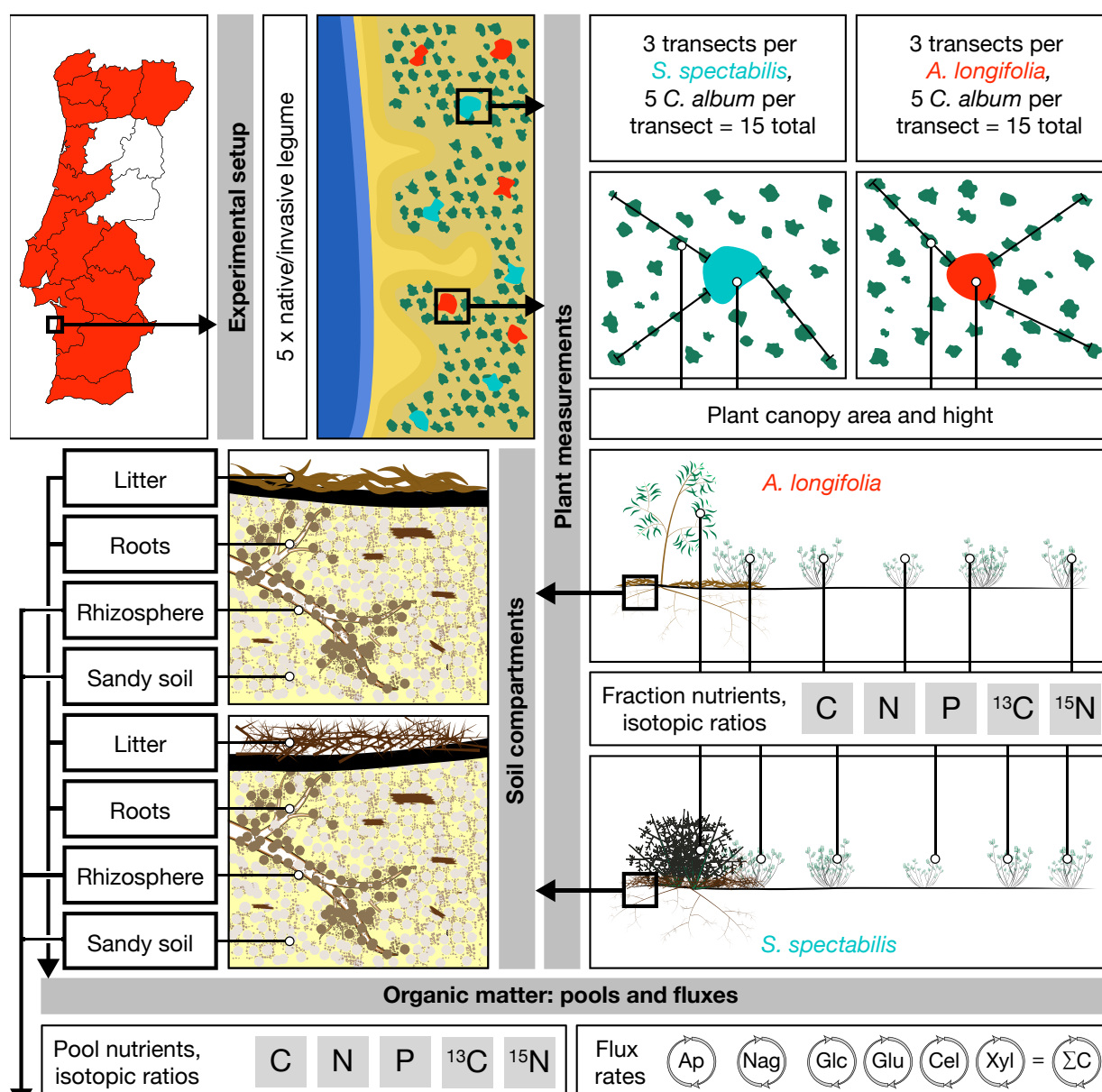


Figure 1.5 Illustrated summary of chapter 3.

Depicted are the localisation of the sampling site (Pinheiro da Cruz, Portugal), the experimental setup in the primary dunes, as well as the analysis the samples were subjected to. Three transects were laid out each around 5 invasive *A. longifolia* and 5 native *S. spectabilis* legume shrubs, starting in their respective canopies next to a *C. album* plant. Canopy sizes and heights of the legumes and *C. album* plants were measured and along each transect, leaves of 5 *C. album* shrubs were sampled, totalling 15 *C. album* samples per legume. Legume foliage was also sampled, three soil samples per legume were taken and separated into the compartments: Litter, rhizosphere, roots and sandy soil. Of these compartments, the rhizosphere, litter and roots were analysed for C, N and P, as well as stable isotopic ratios of C and N. The flux rates of these compartments were also measured using extracellular enzyme analysis. The enzymes measured were: $\Sigma\text{C} = \beta\text{-glucosidase (Glc)} + \beta\text{-xylosidase (Xyl)} + \beta\text{-glucuronidase (Glu)} + \text{cellobiohydrolase (Cel)}$; $\beta\text{-1,4-N-acetylglucosaminidase (Nag)}$ and acid phosphatase (Ap)

on the N distribution into the surrounding *C. album* vegetation (**Figure 1.5**). It was hypothesised that both legumes do have an impact on *C. album* foliar N and $\delta^{15}\text{N}$, with a more pronounced impact by the invasive legume. Furthermore, the increased N availability could putatively induce a systemic N/P imbalance, as N can be fixed from the atmosphere, while P stocks are in general finite in the soil and especially scarce in the oligotrophic dunes. In order to test these hypotheses, the changes in *C. album* foliar N and $\delta^{15}\text{N}$ were modelled using a transect-based breakpoint regression method and the resulting impact boundaries were used to select variables that explain the legume influence. Underneath the legume canopies, soil samples were taken and separated into litter, roots, rhizosphere and sandy soil. The OM concentrations of each compartment were analysed and their C, N and P concentrations determined. Additionally, the potential nutrient flux rates of C, N and P were assessed using fluorogenic artificial substrates to observe EEAs of the litter, roots and the rhizosphere. Finally, values of both the native as well as the invasive legume were pooled together and used in a multivariate approach to select for the most important variables describing the effects they have on the surrounding *C. album* vegetation.

1.4.4 Chapter 4: Following the degradation of invasive species green waste compost in a sandy soil

While chapter 2 and 3 are connected to field observations and sampling, chapter 4 is an entirely lab-based approach (**Figure 1.6**). On the premise of earlier works showing the usefulness of *A. longifolia* compost, standing living

biomass and the litter layer of populations in the Odemira region were sampled, as well as sandy agricultural soil from an adjacent industrial farming site. The chipped biomass, both purely standing biomass (CO treatment), as well as biomass mixed with chipped litter in a 1:10 ratio (CL treatment), were then composted in medium-scaled custom made bioreactors, which maintained the temperature in a mesophilic phase for 40 days. This treatment was applied to kill off the seeds due to the prolonged period of elevated temperature and create a compost potentially ready for agricultural application. Subsequently, both compost types were incorporated into the sampled agricultural soil (2 % w/w), packed into open lid boxes and incubated at constant soil water content for 180 days in laboratory conditions. During the experiment, six time points were taken, three before and three after the addition of $50 \mu\text{g NH}_4^+ \text{g}^{-1}$ soil. As this experiment was supposed to mimic soil response to *A. longifolia* GWC amendment in an industrial agricultural setting, the ammonium applied corresponded to mineral fertilization in a conventional agricultural range. It was hypothesised that the compost applications would increase soil microbial activity, thus leading to a N immobilization in the compost treatments. Also, if N/P imbalance resulting from the increased N availability leads the soil microbial community to increase P acquisition, the compost treatments could help to unlock bound P. As the microbial community in the litter layer below *A. longifolia* should be adapted to the situation of N availability and P scarcity, the CL treatment was hypothesised to exhibit higher EEAs than the biomass compost (CO), given that the microbial community survives composting

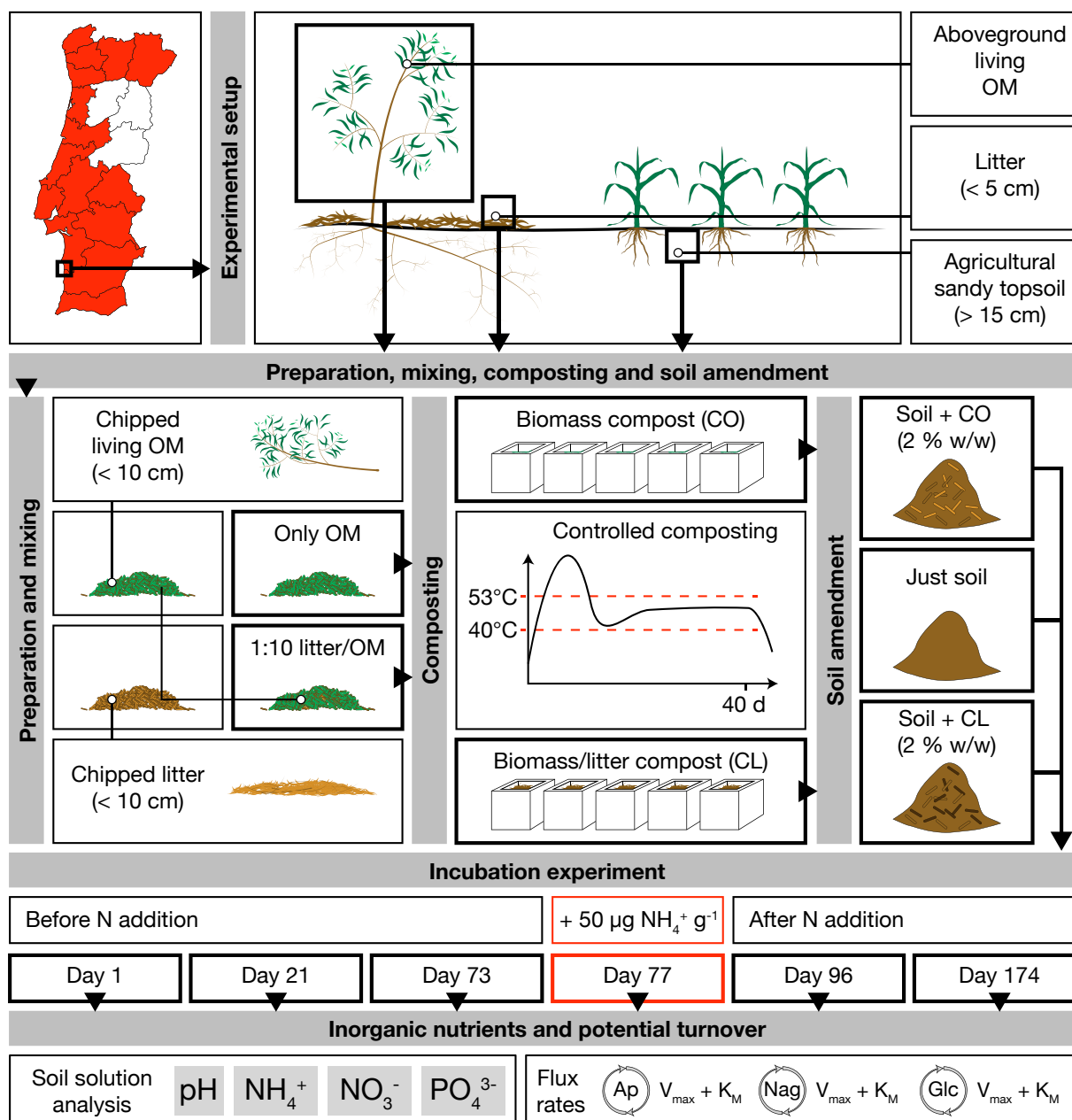


Figure 1.6 Illustrated summary of chapter 4.

Depicted are the localisation of the sampling site (Odemira, Portugal) and the experimental setup. Aboveground plant material as well as the litter layer (top 5 cm) were sampled, plant material and litter was then chipped and composted under controlled conditions (between 40 and 53°C for 40 days). The biomass was either composted alone (CO) or mixed with litter (1:10) before composting (CL). Subsequently, 2 % of the resulting compost was added to an agricultural sandy soil sampled from the same site and incubated in the laboratory. Samples were taken at 6 time points, 3 before and 3 after the addition of $50 \mu\text{g NH}_4^+ \text{g}^{-1}$ and analysed for extracellular enzymes as well as inorganic nutrients found in the soil solution. For the enzymes β -glucosidase (Glc), β -1,4-N-acetylglucosaminidase (Nag) and acid phosphatase (Ap) Michaelis-Menten kinetics were assessed, resulting in K_m as well as V_{\max} values for each enzyme.

1.4.5 Chapter 5: Sustainable urban agriculture using compost and an open-pollinated maize variety

The last chapter is concerned with the application of *A. longifolia* GWC in an actual agricultural setting. While chapter 4 mimics industrial agricultural activities using mineral

fertilizer, this chapter is based on the premise of using GWC with a mix of municipal compost (MC) in order to grow maize solely on the basis of organic soil amendments, without the application of mineral fertilizers (**Figure 1.7**). As this type of organic agriculture is more labour intensive and requires large amounts of

compost additions, it was tested in an urban context, where compost feedstock is more readily available. In this situation however, an economic profile contemplating micro- and macronutrients as well as heavy metals of both the soil and the grain produced is necessary, as in urban and peri-urban farming there is a higher potential for heavy metal pollution.

Indeed, while MC is nutrient rich, it has the drawback of accumulating heavy metals as well. GWC addition can be useful in this context as it is nutrient and heavy metal poor and thus dilutes potential contaminations upon application. Apart from using MC and GWC as compost treatments, two different varieties of maize were tested: An open pollinated

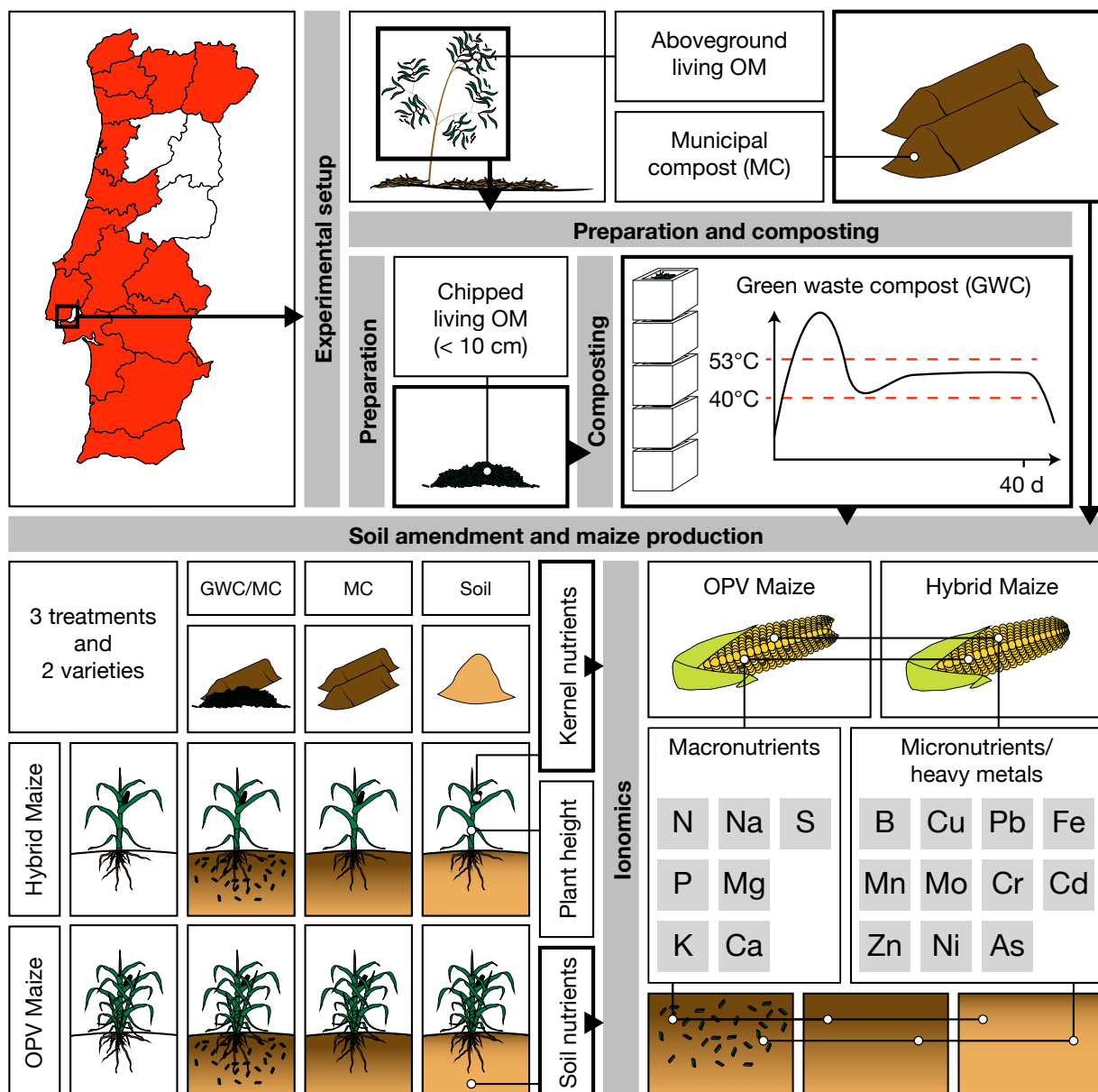


Figure 1.7: Illustrated summary of chapter 5.

Depicted are the localisation of the sampling site (Lisbon, Portugal) and the experimental setup. *A. longifolia* biomass was sampled in the region of Odemira, Portugal, chipped and the composted under controlled conditions (between 40 and 53°C for 40 days). The produced compost, as well as a municipal compost obtained in Lisbon, were used as a soil amendment and treated soil then planted with two varieties of maize: an open pollinated variety (OPV) of maize as well as well as a hybrid variety. Before harvest, plant height and ear number were determined. Maize ears as well as soil samples were collected and dried. Subsequently, maize kernels and soil were analysed for macro and micronutrient concentrations.

variety (OPV) sourced from local seeds, as well as a commercial hybrid variety. It was hypothesised that the OPV is more adapted to organic amendments, as it was grown and selected for a long period of time using solely organic fertilizers before the advent of large-scale mineral fertilization. Furthermore, if it could be shown to produce comparable yields under this type of fertilization regime than a commercial hybrid, it is more suitable for urban small scale agriculture, as its seeds can be reused and thus increase food sovereignty of local populations.

1.5 References

- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S., 2013.** Composting for management and resource recovery of invasive *Acacia* species. *Waste Management & Research*, 31(11), pp.1125-1132.
- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S.R., 2015. (a)** Co-composting of invasive *Acacia longifolia* with pine bark for horticultural use. *Environmental technology*, 36(13), pp.1632-1642.
- Brito, L.M., Reis, M., Mourão, I. and Coutinho, J., 2015. (b)** Use of acacia waste compost as an alternative component for horticultural substrates. *Communications in Soil Science and Plant Analysis*, 46(14), pp.1814-1826.
- Caldwell, B.A., 2005.** Enzyme activities as a component of soil biodiversity: a review. *Pedobiologia*, 49(6), pp.637-644.
- Capinha, C., Essl, F., Seebens, H., Moser, D. and Pereira, H.M., 2015.** The dispersal of alien species redefines biogeography in the Anthropocene. *Science*, 348(6240), pp.1248-1251.
- Carneiro, M., Moreira, R., Gominho, J. and Fabião, A., 2014.** Could control of invasive *Acacias* be a source of biomass for energy under Mediterranean conditions? *Chemical Engineering*, 37.
- Carpenter, S.R. and Bennett, E.M., 2011.** Reconsideration of the planetary boundary for phosphorus. *Environmental Research Letters*, 6(1), p.014009.
- Catford, J.A., Daehler, C.C., Murphy, H.T., Sheppard, A.W., Hardesty, B.D., Westcott, D.A., Rejmánek, M., Bellingham, P.J., Pergl, J., Horvitz, C.C. and Hulme, P.E., 2012.** The intermediate disturbance hypothesis and plant invasions: Implications for species richness and management. *Perspectives in Plant Ecology, Evolution and Systematics*, 14(3), pp.231-241.
- Chytrý, M., Pyšek, P., Wild, J., Pino, J., Maskell, L.C. and Vilà, M., 2009.** European map of alien plant invasions based on the quantitative assessment across habitats. *Diversity and Distributions*, 15(1), pp.98-107.
- Chytrý, M., Wild, J., Pyšek, P., Jarošík, V., Dendoncker, N., Reginster, I., Pino, J., Maskell, L.C., Vilà, M., Pergl, J. and Kühn, I., 2012.** Projecting trends in plant invasions in Europe under different scenarios of future land-use change. *Global Ecology and Biogeography*, 21(1), pp.75-87.
- Cleveland, C.C. and Liptzin, D., 2007.** C: N: P stoichiometry in soil: is there a “Redfield ratio” for the microbial biomass? *Biogeochemistry*, 85(3), pp.235-252.
- Crooks, J.A., 2002.** Characterizing ecosystem-level consequences of biological invasions: the role of ecosystem engineers. *Oikos*, 97(2), pp.153-166.
- Crutzen, P.J., 2002.** Geology of mankind. *Nature*, 415(6867), p.23.
- Conijn, J.G., Bindraban, P.S., Schröder, J.J. and Jongschaap, R.E.E., 2018.** Can our global food system meet food demand within planetary boundaries? *Agriculture, Ecosystems & Environment*, 251, pp.244-256.
- De Vries, W., Kros, J., Kroeze, C. and Seitzinger, S.P., 2013.** Assessing planetary and regional nitrogen boundaries related to food security and adverse environmental impacts. *Current Opinion in Environmental Sustainability*, 5(3-4), pp.392-402.
- deB Richter, D., Bacon, A.R., Brecheisen, Z. and Mobley, M.L., 2015.** Soil in the Anthropocene. In: *IOP Conference Series: Earth and Environmental Science* 25(1), p. 012010. IOP Publishing: England
- Fox, M.D., 2012.** Mediterranean weeds: exchanges of invasive plants between the five Mediterranean regions of the world. In: *Biological invasions in Europe and the Mediterranean Basin*. Springer: Netherlands, pp.179-200
- Dias, T., Crous, C.J., Liberati, D., Munzi, S., Gouveia, C., Ulm, F., Afonso, A.C., Ochoa-Hueso, R., Manrique, E., Sheppard, L. and Martins-Loução, M.A., 2017.** Alleviating nitrogen limitation in Mediterranean maquis vegetation leads to ecological degradation. *Land Degradation & Development*, 28(8), pp.2482-2492.
- Doody, J.P., 2013.** Alien plant invasion. In: *Sand dune conservation, management and restoration*. Springer: Netherlands, pp.177-199.
- Doran, J.W. and Zeiss, M.R., 2000.** Soil health and sustainability: managing the biotic component of soil quality. *Applied soil ecology*, 15(1), pp.3-11.
- Ehrenfeld, J.G., 2003.** Effects of exotic plant invasions on soil nutrient cycling processes. *Ecosystems*, 6(6), pp.503-523.
- Eldridge, S.M., Chan, K.Y., Donovan, N.J., Saleh, F., Orr, L. and Barchia, I., 2018.** Agronomic and economic benefits of green-waste compost for peri-urban vegetable production: implications for food security. *Nutrient cycling in agroecosystems*, 111(2-3), pp.155-173.
- Fei, S., Phillips, J. and Shouse, M., 2014.** Biogeomorphic impacts of invasive species. *Annual review of ecology, evolution, and systematics*, 45.
- Funk, J.L. and Vitousek, P.M., 2007.** Resource-use efficiency and plant invasion in low-resource systems. *Nature*, 446(7139), p.1079.

- Griffin, A.R., Midgley, S.J., Bush, D., Cunningham, P.J. and Rinaudo, A.T., 2011. Global uses of Australian acacias—recent trends and future prospects. *Diversity and Distributions*, 17(5), pp.837-847.
- Große-Stoltenberg, A., Hellmann, C., Thiele, J., Werner, C. and Oldeland, J., 2018. Early detection of GPP-related regime shifts after plant invasion by integrating imaging spectroscopy with airborne LiDAR. *Remote Sensing of Environment*, 209, pp.780-792.
- Hellmann, C., Sutter, R., Rascher, K.G., Máguas, C., Correia, O. and Werner, C., 2011. Impact of an exotic N₂-fixing *Acacia* on composition and N status of a native Mediterranean community. *Acta Oecologica*, 37(1), pp.43-50.
- Hellmann, C., Werner, C. and Oldeland, J., 2016. A spatially explicit dual-isotope approach to map regions of plant-plant interaction after exotic plant invasion. *PloS one*, 11(7), p.e0159403.
- Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H., Lodge, D.M., Loreau, M., Naeem, S. and Schmid, B., 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecological monographs*, 75(1), pp.3-35.
- Hu, C.C., Lei, Y.B., Tan, Y.H., Sun, X.C., Xu, H., Liu, C.Q. and Liu, X.Y., 2019. Plant nitrogen and phosphorus utilization under invasive pressure in a montane ecosystem of tropical China. *Journal of Ecology*, 107(1), pp.372-386.
- Hulme, P.E., Pyšek, P., Jarošík, V., Pergl, J., Schaffner, U. and Vila, M., 2013. Bias and error in understanding plant invasion impacts. *Trends in Ecology & Evolution*, 28(4), pp.212-218.
- Jones, C.G., Lawton, J.H. and Shachak, M., 1994. Organisms as ecosystem engineers. In: *Ecosystem management*. Springer, New York, NY. pp.130-147
- Van Kleunen, M., Dawson, W., Essl, F., Pergl, J., Winter, M., Weber, E., Kreft, H., Weigelt, P., Kartesz, J., Nishino, M. and Antonova, L.A., 2015. Global exchange and accumulation of non-native plants. *Nature*, 525(7567), p.100.
- Klock, M.M., Barrett, L.G., Thrall, P.H. and Harms, K.E., 2016. Differential plant invasiveness is not always driven by host promiscuity with bacterial symbionts. *AoB Plants*, 8.
- Kamutando, C.N., Vikram, S., Kamgan-Nkuekam, G., Makhalanyane, T.P., Greve, M., Le Roux, J.J., Richardson, D.M., Cowan, D. and Valverde, A., 2017. Soil nutritional status and biogeography influence rhizosphere microbial communities associated with the invasive tree *Acacia dealbata*. *Scientific reports*, 7(1), p.6472.
- Kamutando, C.N., Vikram, S., Kamgan-Nkuekam, G., Makhalanyane, T.P., Greve, M., Le Roux, J.J., Richardson, D.M., Cowan, D.A. and Valverde, A., 2019. The Functional Potential of the Rhizospheric Microbiome of an Invasive Tree Species, *Acacia dealbata*. *Microbial ecology*, 77(1), pp.191-200.
- Kulmatiski, A., Beard, K.H. and Heavilin, J., 2012. Plant–soil feedbacks provide an additional explanation for diversity–productivity relationships. *Proceedings of the Royal Society B: Biological Sciences*, 279(1740), pp.3020-3026.
- Le Roux, J.J., Ellis, A.G., van Zyl, L.M., Hosking, N.D., Keet, J.H. and Yannelli, F.A., 2018. Importance of soil legacy effects and successful mutualistic interactions during Australian acacia invasions in nutrient-poor environments. *Journal of Ecology*, 106(5), pp.2071-2081.
- Liao, C., Peng, R., Luo, Y., Zhou, X., Wu, X., Fang, C., Chen, J. and Li, B., 2008. Altered ecosystem carbon and nitrogen cycles by plant invasion: a meta-analysis. *New phytologist*, 177(3), pp.706-714.
- Lowe, S., Browne, M., Boudjelas, S. and De Poorter, M., 2000. 100 of the world's worst invasive alien species: a selection from the global invasive species database, 12. Auckland: Invasive Species Specialist Group.
- Mace, G.M., Reyers, B., Alkemade, R., Biggs, R., Chapin III, F.S., Cornell, S.E., Díaz, S., Jennings, S., Leadley, P., Mumby, P.J. and Purvis, A., 2014. Approaches to defining a planetary boundary for biodiversity. *Global Environmental Change*, 28, pp.289-297.
- Máguas, C., Rascher, K.G., Martins-Loução, A., Carvalho, P., Pinho, P., Ramos, M., Correia, O. and Werner, C., 2011. Responses of woody species to spatial and temporal ground water changes in coastal sand dune systems. *Biogeosciences*, 8(12), pp.3823-3832.
- Manzoni, S., Trofymow, J.A., Jackson, R.B. and Porporato, A., 2010. Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. *Ecological Monographs*, 80(1), pp.89-106.
- Marchante, H., Marchante, E. and Freitas, H., 2003. Invasion of the Portuguese dune ecosystems by the exotic species *Acacia longifolia* (Andrews) Willd.: effects at the community level. In: *Plant invasions: ecological threats and management solutions*, Backhuys Publishers, Leiden, The Netherlands, pp.75-85.
- Marchante, H.S., Marchante, E.M., Buscardo, E., Maia, J. and Freitas, H., 2004. Recovery potential of dune ecosystems invaded by an exotic *Acacia* species (*Acacia longifolia*). *Weed Technology*, pp.1427-1433.

- Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2007.** Soil microbial activity in dune ecosystems in Portugal invaded by *Acacia longifolia*. Plant invasions: human perception, ecological impacts and management. Backhuys Publishers, Leiden, The Netherlands, pp.247-257.
- Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2008. (a)** Invasive *Acacia longifolia* induce changes in the microbial catabolic diversity of sand dunes. Soil Biology and Biochemistry, 40(10), pp.2563-2568.
- Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2008. (b)** Short-and long-term impacts of *Acacia longifolia* invasion on the belowground processes of a Mediterranean coastal dune ecosystem. Applied Soil Ecology, 40(2), pp.210-217.
- Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2009.** Soil recovery after removal of the N₂-fixing invasive *Acacia longifolia*: consequences for ecosystem restoration. Biological Invasions, 11(4), pp.813-823.
- Marchante, H., Freitas, H. and Hoffmann, J.H., 2010.** Seed ecology of an invasive alien species, *Acacia longifolia* (Fabaceae), in Portuguese dune ecosystems. American journal of botany, 97(11), pp.1780-1790.
- Marchante, H., Freitas, H. and Hoffmann, J.H., 2011.** Post-clearing recovery of coastal dunes invaded by *Acacia longifolia*: is duration of invasion relevant for management success?. Journal of Applied Ecology, 48(5), pp.1295-1304.
- Marchante, H., Marchante, E., Freitas, H. and Hoffmann, J.H., 2015.** Temporal changes in the impacts on plant communities of an invasive alien tree, *Acacia longifolia*. Plant ecology, 216(11), pp.1481-1498.
- Mariotte, P., Mehrabi, Z., Bezemer, T.M., De Deyn, G.B., Kulmatiski, A., Drigo, B., Veen, G.C., Van der Heijden, M.G. and Kardol, P., 2018.** Plant–soil feedback: bridging natural and agricultural sciences. Trends in ecology & evolution, 33(2), pp.129-142.
- Martin, F., Díez, J., Dell, B. and Delaruelle, C., 2002.** Phylogeography of the ectomycorrhizal *Pisolithus* species as inferred from nuclear ribosomal DNA ITS sequences. New Phytologist, 153(2), pp.345-357.
- Miller, J.T., Murphy, D.J., Brown, G.K., Richardson, D.M. and González-Orozco, C.E., 2011.** The evolution and phylogenetic placement of invasive Australian *Acacia* species. Diversity and Distributions, 17(5), pp.848-860.
- Mollot, G., Pantel, J.H. and Romanuk, T.N., 2017.** The effects of invasive species on the decline in species richness: a global meta-analysis. Advances in Ecological Research, 56, pp. 61-83.
- Morais, M.C., Panuccio, M.R., Muscolo, A. and Freitas, H., 2012.** Salt tolerance traits increase the invasive success of *Acacia longifolia* in Portuguese coastal dunes. Plant Physiology and Biochemistry, 55, pp.60-65.
- Morris, T.L., Esler, K.J., Barger, N.N., Jacobs, S.M. and Cramer, M.D., 2011.** Ecophysiological traits associated with the competitive ability of invasive Australian acacias. Diversity and Distributions, 17(5), pp.898-910.
- Ngorima, A. and Shackleton, C.M., 2019.** Livelihood benefits and costs from an invasive alien tree (*Acacia dealbata*) to rural communities in the Eastern Cape, South Africa. Journal of environmental management, 229, pp.158-165.
- Penuelas, J., Poulter, B., Sardans, J., Ciais, P., Van Der Velde, M., Bopp, L., Boucher, O., Godderis, Y., Hinsinger, P., Llusia, J. and Nardin, E., 2013.** Human-induced nitrogen–phosphorus imbalances alter natural and managed ecosystems across the globe. Nature communications, 4, p.2934.
- Peperkorn, R., Werner, C. and Beyschlag, W., 2005.** Phenotypic plasticity of an invasive acacia versus two native Mediterranean species. Functional Plant Biology, 32(10), pp.933-944.
- Philippot, L., Raaijmakers, J.M., Lemanceau, P. and Van Der Putten, W.H., 2013.** Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology, 11(11), p.789.
- Pritsch, K., Esperschuetz, J., Haesler, F., Raidl, S., Winkler, B. and Schlöter, M., 2009.** Structure and activities of ectomycorrhizal and microbial communities in the rhizosphere of *Fagus sylvatica* under ozone and pathogen stress in a lysimeter study. Plant and soil, 323(1-2), pp.97-109.
- Rascher, K.G., Große-Stoltenberg, A., Máguas, C., Meira-Neto, J.A.A. and Werner, C., 2011.** *Acacia-longifolia* invasion impacts vegetation structure and regeneration dynamics in open dunes and pine forests. Biological Invasions, 13(5), pp.1099-1113.
- Rascher, K.G., Hellmann, C., Máguas, C. and Werner, C., 2012.** Community scale ¹⁵N isoscapes: tracing the spatial impact of an exotic N₂-fixing invader. Ecology letters, 15(5), pp.484-491.
- Reeve, J.R., Hoagland, L.A., Villalba, J.J., Carr, P.M., Atucha, A., Cambardella, C., Davis, D.R. and Delate, K., 2016.** Organic farming, soil health, and food quality: considering possible links. Advances in Agronomy 37, pp. 319-367.
- Reyes-Torres, M., Oviedo-Ocaña, E.R., Dominguez, I., Komilis, D. and Sánchez, A., 2018.** A systematic review on the composting of green waste: Feedstock quality and optimization strategies. Waste management.

- Ricciardi, A., Blackburn, T.M., Carlton, J.T., Dick, J.T., Hulme, P.E., Iacarella, J.C., Jeschke, J.M., Liebhold, A.M., Lockwood, J.L., MacIsaac, H.J. and Pyšek, P., 2017.** Invasion science: a horizon scan of emerging challenges and opportunities. *Trends in Ecology & Evolution*, 32(6), pp.464-474.
- Richardson, D.M., Pyšek, P., Rejmánek, M., Barbour, M.G., Panetta, F.D. and West, C.J., 2000.** Naturalization and invasion of alien plants: concepts and definitions. *Diversity and distributions*, 6(2), pp.93-107.
- Richardson, A.E., Barea, J.M., McNeill, A.M. and Prigent-Combaret, C., 2009.** Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and soil*, 321(1-2), pp.305-339.
- Richardson, D.M. and Rejmánek, M., 2011.** Trees and shrubs as invasive alien species—a global review. *Diversity and distributions*, 17(5), pp.788-809.
- Richardson, D.M., Carruthers, J., Hui, C., Impson, F.A., Miller, J.T., Robertson, M.P., Rouget, M., Le Roux, J.J. and Wilson, J.R., 2011.** Human-mediated introductions of Australian acacias—a global experiment in biogeography. *Diversity and Distributions*, 17(5), pp.771-787.
- Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin III, F.S., Lambin, E.F., Lenton, T.M., Scheffer, M., Folke, C., Schellnhuber, H.J. and Nykvist, B., 2009.** A safe operating space for humanity. *Nature*, 461(7263), p.472.
- Rodríguez-Echeverría, S., Crisóstomo, J.A. and Freitas, H., 2007.** Genetic diversity of rhizobia associated with *Acacia longifolia* in two stages of invasion of coastal sand dunes. *Applied and environmental microbiology*, 73(15), pp.5066-5070.
- Rodríguez-Echeverría, S., Crisóstomo, J.A., Nabais, C. and Freitas, H., 2009.** Belowground mutualists and the invasive ability of *Acacia longifolia* in coastal dunes of Portugal. *Biological invasions*, 11(3), pp.651-661.
- Rodríguez-Echeverría, S., 2010.** Rhizobial hitchhikers from Down Under: invasional meltdown in a plant-bacteria mutualism?. *Journal of Biogeography*, 37(8), pp.1611-1622.
- Rout, M.E. and Callaway, R.M., 2009.** An invasive plant paradox. *Science*, 324(5928), pp.734-735.
- Salman, A.H.P.M. and Strating, K.M., 1992.** European coastal dunes and their decline since 1900. European Union for Coastal Conservation (EUCC), Leiden, The Netherlands, pp.439-449.
- Sardans, J. and Peñuelas, J., 2012.** The role of plants in the effects of global change on nutrient availability and stoichiometry in the plant-soil system. *Plant physiology*, 160(4), pp.1741-1761.
- Schwarz, C., Ysebaert, T., Vandenbruwaene, W., Temmerman, S., Zhang, L. and Herman, P.M., 2016.** On the potential of plant species invasion influencing bio-geomorphologic landscape formation in salt marshes. *Earth Surface Processes and Landforms*, 41(14), pp.2047-2057.
- Scotti, R., Pane, C., Spaccini, R., Palese, A.M., Piccolo, A., Celano, G. and Zaccardelli, M., 2016.** On-farm compost: a useful tool to improve soil quality under intensive farming systems. *Applied soil ecology*, 107, pp.13-23.
- Shackleton, R.T., Shackleton, C.M. and Kull, C.A., 2019.** The role of invasive alien species in shaping local livelihoods and human well-being: A review. *Journal of environmental management*, 229, pp.145-157.
- Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E. and Gartner, T.B., 2008.** Stoichiometry of soil enzyme activity at global scale. *Ecology letters*, 11(11), pp.1252-1264.
- Sinsabaugh, R.L., Hill, B.H. and Shah, J.J.F., 2009.** Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature*, 462(7274), p.795.
- Six, J., Conant, R.T., Paul, E.A. and Paustian, K., 2002.** Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and Soil*, 241(2), pp.155-176.
- Souza-Alonso, P., Rodríguez, J., González, L. and Lorenzo, P., 2017.** Here to stay. Recent advances and perspectives about *Acacia* invasion in Mediterranean areas. *Annals of forest science*, 74(3), p.55.
- Souza-Alonso, P., Puig, C.G., Pedrol, N., Freitas, H., Rodríguez-Echeverría, S. and Lorenzo, P., 2018.** Exploring the use of residues from the invasive *Acacia* sp. for weed control. *Renewable Agriculture and Food Systems*, pp.1-12.
- Steffen, W., Richardson, K., Rockström, J., Cornell, S.E., Fetzer, I., Bennett, E.M., Biggs, R., Carpenter, S.R., De Vries, W., De Wit, C.A. and Folke, C., 2015.** Planetary boundaries: Guiding human development on a changing planet. *Science*, 347(6223), p.1259855.
- Unkovich, M., 2013.** Isotope discrimination provides new insight into biological nitrogen fixation. *New Phytologist*, 198(3), pp.643-646.
- Vilà, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarošík, V., Maron, J.L., Pergl, J., Schaffner, U., Sun, Y. and Pyšek, P., 2011.** Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. *Ecology letters*, 14(7), pp.702-708.

Wassen, M.J., Venterink, H.O., Lapshina, E.D. and Tanneberger, F., 2005. Endangered plants persist under phosphorus limitation. *Nature*, 437(7058), p.547.

Werner, C., Zumkier, U., Beyschlag, W. and Máguas, C., 2010. High competitiveness of a resource demanding invasive acacia under low resource supply. *Plant ecology*, 206(1), pp.83-96.

Zalasiewicz, J., Waters, C., Summerhayes, C. and Williams, M., 2018. The Anthropocene. *Geology Today*, 34(5), pp.177-181.

Zalidis, G., Stamatiadis, S., Takavakoglou, V., Eskridge, K. and Misopolinos, N., 2002. Impacts of agricultural practices on soil and water quality in the Mediterranean region and proposed assessment methodology. *Agriculture, Ecosystems & Environment*, 88(2), pp.137-146.

Zhang, P., Li, B., Wu, J. and Hu, S., 2019. Invasive plants differentially affect soil biota through litter and rhizosphere pathways: a meta-analysis. *Ecology letters*, 22(1), pp.200-210.

Chapter 2

How to outgrow your native neighbour? Belowground changes under native shrubs at an early stage of invasion

Ulm, F., Jacinto, J., Cruz, C. and Máguas, C., 2017. How to outgrow your native neighbour? Belowground changes under native shrubs at an early stage of invasion. *Land Degradation & Development*, 28(8), pp.2380-2388.

2.1 Abstract

While it is acknowledged that invasive species are a global driver of land degradation, their effects are often only noticed when the invasion has been going on for a while. However, early stage processes must play a fundamental role in plant establishment until invasive plants are able to outgrow the native vegetation. In ten plots of 100 m² each, we tested the hypothesis that belowground properties are associated with early invasion processes aboveground. We examined the early stage of invasion by a woody legume (*Acacia longifolia*), growing in the canopy of native dune shrubs (*Corema album*) as a model system in oligotrophic primary dunes in southern Portugal.

Biomass under canopies of invaded and non-invaded *C. album* shrubs as well as organic matter (OM) distribution in various soil fractions was measured. In accordance with our hypothesis, *A. longifolia* presence was related to increased *C. album* foliar $\delta^{15}\text{N}$, a proxy for nitrogen derived from the invasive legume.

Under invaded canopies, root and rhizosphere biomass were higher, as was OM in the silt-clay fraction. Also, $\delta^{15}\text{N}$ of the OM in the silt-clay fraction under invaded canopies was enriched, while $\delta^{13}\text{C}$ was depleted. Finally, we found that the ratio between OM in the biotic versus soil compartment could be a good early indicator for invasion.

These findings suggest that even when above-ground invasion pressure on the system is low, it is imperative for ecosystem conservation to remove young plants, as they might alter soil functioning already at an early stage of invasion.

2.2 Introduction

The introduction of invasive plant species into novel habitats leads to the degradation of many ecosystems worldwide and poses a fundamental challenge to the conservation of biodiversity (Simberloff *et al.*, 2013). One of the major global gateways for plant invasion is the anthropogenic introduction of non-native plant species for dune stabilization, which might then turn invasive over time (Doody, 2013). However, while some invasive plant species can be more efficient in dune stabilization than native plants, reduction in native plant biodiversity and abundance will ultimately decrease the resilience of these ecosystems (Charbonneau *et al.*, 2017).

Oligotrophic systems, such as dunes, are very vulnerable to plant invasions, for example due to resource competition between native and invading plant species (Martínez and García-Franco, 2008). Especially woody legumes have strong effects on oligotrophic ecosystems due to their longevity and biomass production (Richardson *et al.*, 2014), as well as their ability to fix atmospheric nitrogen (N), which makes them prolific ecosystem engineers (Rundel *et al.*, 2014). As dunes are naturally characterized by intermediate regular disturbance, they are particularly vulnerable to the biogeomorphic effects of ecosystem engineers, for example soil fixation by roots and soil organic matter (OM) accumulation (Fei *et al.*, 2014). Thus, belowground processes play a crucial role in the invasion process (Nuñez and Dickie, 2014) and plant–soil interactions are particularly important in early facilitation processes as found in oligotrophic systems (Van der Putten *et al.*, 2013).

Very little is known about the early stage of plant invasion with low plant densities (Elgersma and Ehrenfeld, 2011). An important factor to consider in dune systems is facilitation of seedlings by nurse plants (Armas and Pugnaire, 2005), and positive plant–plant interactions seem to be more dominant than competition at an early stage of these ecosystems (Martínez and García-Franco, 2008). However, the shift from facilitation to competition is gradual and dependent on the site of interaction. For example, it was found that the interaction between nursery shrubs and tree seedlings might range from facilitation under environmental stress to competition in less stressful conditions (Muhammed *et al.*, 2013).

In the Portuguese dune systems, one of the most prolific invasive species is *Acacia longifolia*, with highly detrimental impacts on native vegetation and soil (Marchante *et al.*, 2008). The most severe impacts on invaded ecosystems are a strong decline in native species richness (Marchante *et al.*, 2015), as well as the establishment of an abundant seed bank of *A. longifolia*, accompanied by soil eutrophication (Marchante *et al.*, 2008). The invasion process is presumed to start in the nutrient patches developed underneath existing native vegetation, which act as nursery plants (Peperkorn, 2005) and where *A. longifolia* grows more predominant than in the open soil. As *A. longifolia* is highly competitive under sufficient nutrient supply, as well as in nutrient poor conditions due to its ability to fix N (Werner *et al.*, 2010), it starts to outgrow its native nursery plant.

Subsequently, the ecosystem engineering process continues and changes on soil level strengthen invasion (Marchante *et al.*, 2008) escalating the invasion further along time (Marchante *et al.*, 2015). Medium-term to long-term effects on soil functioning include changes in carbon (C), N (Marchante *et al.*, 2008) and phosphorus (P) pools (Ulm *et al.*, 2017), which are mainly related to altered soil catabolism, as well as a thick litter layer (Marchante *et al.*, 2008) and increased root mass (Morris *et al.*, 2011). These processes ultimately shift the primary dune system from an open vegetation structure of native shrubs to almost exclusive monospecific stands of medium-sized *A. longifolia* trees (Rascher *et al.*, 2012).

Interestingly, while the long-term effects of *A. longifolia* on the native vegetation are clearly negative, it has been shown that native shrubs like the Ericaceae *Corema album* can initially benefit from the increased N availability (Hellmann *et al.*, 2011; Rascher *et al.*, 2012). Using tracers such as foliar $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, it has been shown that *C. album* exhibits higher growth rates and foliar N content with *A. longifolia* presence (Hellmann *et al.*, 2011). These effects are exceeding *A. longifolia* canopy limits (Rascher *et al.*, 2012) and might have secondary effects on *C. album*, such as increased photosynthetic capacity through better plant N nutrition (Hellmann *et al.*, 2016). On the longer term, however, *A. longifolia* dominates *C. album* due to high growth rates and phenotypic plasticity, consequently increasing competition for resources like water (Hellmann *et al.*, 2016) or P (Ulm *et al.*, 2017). Thus, in the invasion process, the coexistence of both plants shifts from facilitation of *C. album* to

co-facilitation by both *A. longifolia* and *C. album* and culminates in a competition, which ultimately ends favourably for the invader.

While the later stages of invasion, co-facilitation and competition, are well studied (Hellmann *et al.*, 2011; Marchante *et al.*, 2008, 2015), the initial phase of invasion, changing facilitation to co-facilitation and beginning competition, is less clear. Most of the competitive advantage of the invasive species can be related to its biomass production, for example its litter layer. However, at the beginning of the dune invasion, biomass of *A. longifolia* is still very low and no litter layer has yet developed. Thus, self-facilitation by its own above-ground biomass is therefore not yet viable. The invader nevertheless outgrows its native nursery shrub, which, as no invasive above-ground material is available yet, pinpoints to belowground processes being changed by the presence of the invasive species. Belowground biomass accumulation is a typical process for ecosystem engineering plants (Jones *et al.*, 1996) and apart from roots, also the rhizosphere increases underneath large *A. longifolia* specimen (Ulm *et al.*, 2017), as does soil OM content (Hellmann *et al.*, 2011). Assessing processes occurring in the rhizosphere can be crucial to understand plant invasion (Philippot *et al.*, 2013), as the rhizosphere is the main site for plant-microbe interactions that mediate OM turnover rates (Ulm *et al.*, 2017). Also, soil OM increase has been shown to be critical in invasion, as it creates a positive feedback loop between plant and soil (Marchante *et al.*, 2008), thus exacerbating the invasion processes. Not only soil OM pools have been shown to be important but also their P concentrations, as *A. longifolia* generates an N/P imbalance in

plant biomass over time as it increases N input while demanding P for constant growth (Ulm *et al.*, 2017). Also, OM might accumulate through stabilization in certain soil fractions, for example the silt-clay fraction (Six *et al.*, 2002), which is known to increase after plant invasions in other dune-like systems (e.g. salt marshes) (Schwarz *et al.*, 2016). However, not only the quantity of this fraction but also its quality has to be considered, for example C:N ratios or $\delta^{13}\text{C}$, which can give information about turnover and residence times (e.g. McClaran *et al.*, 2008).

In the work presented here, we first wanted to confirm that *A. longifolia* plants without developed litter layer have an effect on *C. album* shrubs, using *C. album* foliar $\delta^{15}\text{N}$ as an ecological tracer. Subsequently, we aimed to connect aboveground responses with below-ground changes, focussing especially on plant biomass and soil OM. Due to earlier observations (Ulm *et al.*, 2017), we expected differences in biomass N and P concentrations. Furthermore, we postulated differences in the silt-clay fraction of the soil under invaded plants as a proxy for stabilized soil OM. While we assumed the overall quantity of this fraction to be miniscule due to the extremely oligotrophic nature of this sand dune system, we wanted to explore its potential to indicate biomass flows and soil mechanism changes during early invasion. Lastly, we explored the ratio of belowground plant biomass versus soil OM as a proxy for invasion impact.

2.3 Materials and Methods

2.3.1 Study site and sampling design

Sampling took place from 12 to 28 July 2013 in the coastal sand dune ecosystem of Pinheiro da Cruz, Portugal (38°15.2'N, 8°45.8'W), which is invaded by the non-native leguminous tree *Acacia longifolia* (Andrews) Willd. (Fabaceae). The site is located in the primary dunes, which are characterized by poor arenosols (FAO classification) and an open vegetation structure (Rascher *et al.*, 2012), which is dominated by *Corema album* (L.) D. Don (Ericaceae), a shrub endemic to the Iberian Peninsula.

Sampling took place in a 10,000 m² area, using ten plots of 100 m² each, laid out randomly, but more than 50 m apart and containing at least one *A. longifolia* plant growing inside the canopy of a *C. album* shrub. Already developed stands or shrubs were not considered, as the aim was to analyse plants in a very early stage of invasion, for example < 2 m height and without any aboveground litter. After counting all *A. longifolia* and *C. album* plants in each plot, their canopy sizes were estimated by an ellipsoid and their volumes estimated assuming an upper half spheroid shape ($\frac{4}{3} * c_1 * c_2 * \pi * h * 0.5$), following recommendations for shrubs in arid landscapes by Ludwig *et al.* (1975). Per plot, soil was sampled with a metal tube (8.5 cm diameter, 19.5 cm height) once within the canopy of a *C. album* plant growing far away (> 5 m) from any *A. longifolia* plant (- Inf) and once within the canopy of a *C. album* plant with an *A. longifolia* plant growing inside its canopy (+ Inf). Bulk samples were separated into four fractions: sand, roots, rhizosphere and large OM particles (>

2 mm), which were termed coarse OM. The sand fraction was removed from the bulk-agglomerated rhizosphere/root in the core and sieved through a 2 mm sieve. Rhizosphere was separated from roots by gently shaking the adhered particles off the roots and subsequently treating them similar to the soil as described in Ulm *et al.* (2017). Parts of all fractions were oven-dried at 60°C until constant weight and 500 mg subsequently ashed in a muffle furnace (600°C, 24 h) to determine OM by the loss on ignition method.

2.3.2 Soil fractionation and phosphorus determination

One hundred grams of dry, sieved (2 mm) soil sample was fractionated by using an automatic vibratory sieve shaker (Fritsch Analysette 3, Fritsch, Idar-Oberstein, Germany) and two sieve sizes: 425 and 63 µm. Resulting fractions were termed > 425, > 63 and < 63 µm. The sieving programme used had an interval time of 10 s, with 3 min sieving time and an amplitude of 3 mm; mean sample recovery rate of the procedure was more than 99.84%. Subsequently, each fraction was analysed for OM and total phosphorus (P). Only the fraction < 63 µm was analysed for % C, % N, δ¹⁵N and δ¹³C, as the other fractions, similar to total soil OM, were below detection limit (Rascher *et al.*, 2012).

For total P of soil and OM, samples were ignited, subsequently acid-extracted (HCl, 1 M) and a malachite-green based microscale method employed as described by D'Angelo *et al.* (2001). The colorimetric method was executed in 250-µl 96-well flat bottom microti-

ter plates and analysed in a microplate reader (Rainbow, Tecan, Männedorf, Switzerland). For each single assay, a separate triplicate calibration curve was produced with KH_2PO_4 as a serial dilution in ultrapure water.

2.3.3 Elemental analysis and isotopic ratios

Dry samples of foliage and the soil fraction $< 63 \mu\text{m}$ were ground to a fine powder in a ball mill (Retsch, Haan, Germany). Powder ($5 \pm 0.2 \text{ mg}$) was weighted into tin capsules and analysed at the Stable Isotopes and Instrumental Analysis Facility of the Centre for Ecology, Evolution and Environmental Change, University of Lisbon – Portugal. The $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios in the samples were determined by continuous flow isotope-ratio mass spectrometry on an Isoprime (GV, UK) stable isotope ratio mass spectrometer coupled to an EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. All values are given in δ notation. The standards used were International Atomic Energy Agency N1 (IAEA-N1) and U.S. Geological Survey-35 for N isotope ratio and IAEA-CH6 and IAEA-CH7 for C isotope ratio; $\delta^{15}\text{N}$ results were referred to Air and $\delta^{13}\text{C}$ to PeeDee Belemnite. Precision of the isotope ratio analysis, calculated by using values from 6 to 9 replicates of laboratory standard material interspersed among samples in every batch analysis, was $\leq 0.2\%$.

2.3.4 Statistical analysis

All tests were performed with the package ‘stats’ by using R version 3.3.2 (R Core Team, 2016). Density distribution of plant volume was projected using the *density()* function to compute kernel density estimates. Group wise comparisons were tested for using the pairwise Wilcoxon rank sum test with Holm correction; if only two groups were compared, a simple Wilcoxon rank sum test was used. Linear relationships were tested against heteroscedasticity by using the Breusch-Pagan test (package ‘lmtest’), and the residuals were tested for normality using the Shapiro–Wilk normality test. Multiple factor analysis (MFA) as described by Borcard *et al.* (2011) was used as an exploratory, symmetrical method to test for relationships between the different variable subsets using the function *MFA()* (package ‘FactoMineR’). MFA is fundamentally a principal component analysis on the correlation matrix of each subset, which are then weighed and subsequently combined in a global principal component analysis. Variables used were grouped in OM pools (roots, rhizosphere, coarse OM and OM of soil fractions: > 425 , > 63 , $< 63 \mu\text{m}$), characteristics of the soil fraction $< 63 \mu\text{m}$ (N:P ratio, C:N ratio, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$) and foliar characteristics and plant size (N:P ratio, C:N ratio, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$, plant volume). RV coefficients were calculated for the variable sets used in the MFA, as well as on the two variables resulting from the matrix of plant OM contribution to total OM and total P. RV coefficients are a multivariate version of Pearson r^2 and thus always positive. RV coefficients were then tested for significance using a permutational analysis from the function *coeffRV()* (package ‘FactoMineR’).

2.4 Results

2.4.1 Invasion pattern and aboveground plant characteristics

Within the 10,000 m² covered here, ten young *A. longifolia* plants were found; thus, from the 170 *C. album* specimen examined, 5.88% were invaded. The density distribution of plant sizes by volume (**figure 2.1**) showed no specific *C. album* volume that was more prone to invasion in this area. This was further tested by using a Kolmogorov–Smirnov test, which rejected a significant difference between distributions ($n_{C. album (total)} = 170$, $n_{C. album (invaded)} = 10$, $D = 0.282$, $p\text{-value} = 0.438$).

The invasive *A. longifolia* plants studied here were marginally higher than the native *C. album* shrubs but had smaller canopy sizes, and there were no significant differences found between plant volumes (**table 2.1**). As *A. longifolia* was growing within *C. album*, the addition of both plant volumes was used as a conservative estimate of joined plant size, which does not significantly differ from the plant volume

of non-invaded *C. album* (Wilcox test, $n = 10$ for combined *A. longifolia* and *C. album* and $n = 9$ for non-invaded *C. album*, $p = 0.90$). Foliar N concentration was ~3-fold higher in the invasive plant, while total C was significantly lower and C:N ratios were about half of the

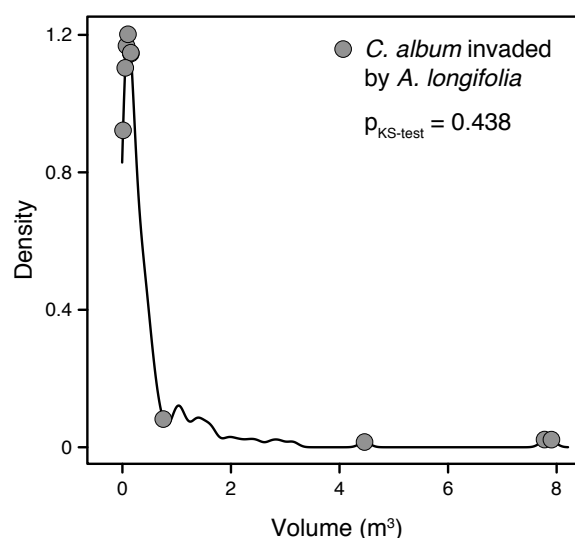


Figure 2.1 Kernel density estimation of *Corema album* size, expressed in volume (m³).

The grey spheres show *C. album* shrubs invaded by *Acacia longifolia*. Distribution was estimated from $n = 170$ plants, with ten plants being invaded. The p -value depicts results of a Kolmogorov–Smirnov test for a significant difference between distributions of invaded and non-invaded *C. album* shrubs.

Table 2.1 Differences between growth form and foliar nutrient content as well as stable isotope values of *Acacia longifolia*, invaded (+ Inv) and non-invaded *Corema album* (- Inv).

The letters indicate significant differences resulting from a pairwise Wilcoxon rank sum test with Holm correction, with $n = 10$ for *A. longifolia*, $n = 10$ for *C. album* (+ Inv) and $n = 9$ for *C. album* (- Inv). Significant differences between groups are shown in bold.

		<i>A. longifolia</i>	<i>C. album</i> (- Inv)	<i>C. album</i> (+ Inv)
Growth form	Height (m)	1.2 (0.2) ^a	0.7 (0.1) ^a	0.7 (0.1) ^a
	Canopy (m²)	0.6 (0.1)^a	3.5 (1.4)^b	3.7 (1.6)^{ab}
	Volume (m ³)	0.6 (0.2) ^a	1.5 (0.6) ^a	2.5 (1.2) ^a
Foliar nutrient concentration and stable isotope values	$\delta^{15}\text{N}$	-2 (0.2)^a	-7.7 (0.6)^b	-6.1 (0.6)^c
	$\delta^{13}\text{C}$	-26.5 (0.5) ^a	-25.8 (0.2) ^a	-25.8 (0.3) ^a
	% N	1.7 (0.2)^a	0.6 (0)^b	0.7 (0.1)^b
	% C	48 (0.9)^a	53.4 (0.2)^b	52.9 (0.3)^b
	‰ P	0.5 (0.1) ^a	0.6 (0.1) ^a	0.6 (0.1) ^a
	C:N	46 (19.3)^a	95.3 (3.8)^b	81.5 (5.8)^b
	N:P	37.5 (6.7)^a	10.2 (0.9)^b	13 (1.8)^b

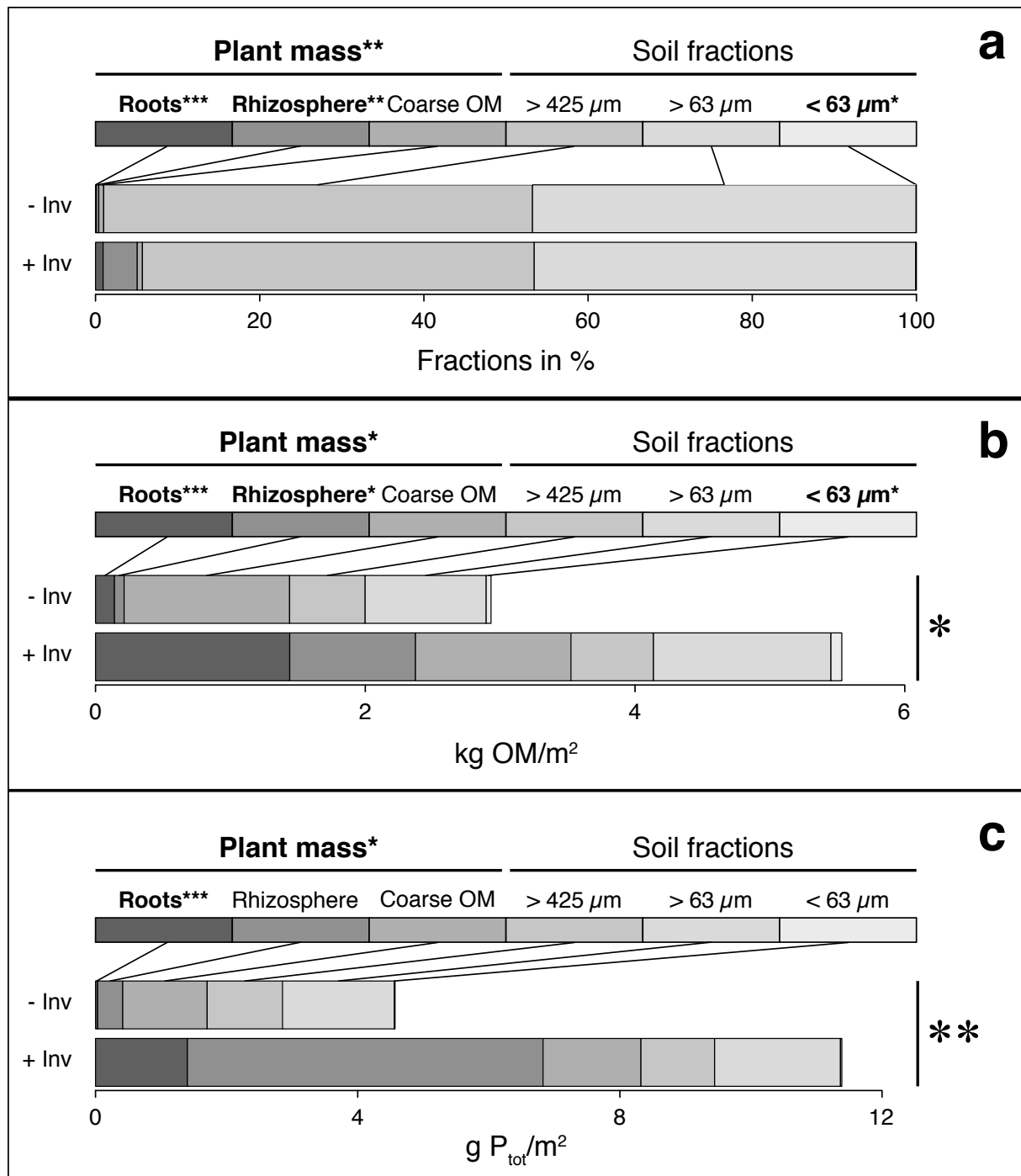


Figure 2.2 Belowground mass pools underneath non-invaded (- Inv) and invaded (+ Inv) *Corema album* plants.
(a) The mass balance in dry weight
(b) The mass balance of organic matter (OM) per m²
(c) The mass balance of total phosphorus (P_{tot}) per m². All these values were measured in the topsoil (20 cm).
The asterisks highlight significant differences, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ (Wilcoxon rank sum test, $n = 10$ for + Inv; $n = 9$ for - Inv).

values observed in native *C. album* shrubs (**table 2.1**). The foliage of *A. longifolia* exhibited the least depleted $\delta^{15}\text{N}$ values, while foliage of non-invaded *C. album* was significantly more depleted and values of invaded *C. album* were in between. While P content is similar in both species, *A. longifolia* exhibited about 3 times higher N:P ratios (**table 2.1**).

2.4.2 Belowground mass, organic matter and P pools in invaded and non-invaded *C. album*

Various belowground changes were found between invaded and non-invaded *C. album* plants (**figure 2.2**). As the soil cores taken had a length of 19.5 cm, only topsoil changes were observed here. Also, the changes in root mass, OM and total P (P_{tot}) pools were not attributed to a singular plant but are rather a mix of both *A. longifolia* and *C. album* mass. The presence of *A. longifolia* plants in the canopy of *C. album* was found to significantly increase

root mass and OM by a factor of 10, as well as P_{tot} by a factor of 40. There was also a 14-fold increase of rhizosphere mass and OM under invaded *C. album* canopies. This significantly increased belowground plant biomass in invaded canopies in general (5.5-fold), as well as plant biomass contribution to total OM (2.4-fold) and P_{tot} (4.9-fold). Also, taking all compartments (plant mass and soil fractions) together, total OM and P_{tot} were higher underneath invaded plant canopies. Invaded plant canopies also showed marginally higher P_{tot} values in the rhizosphere compartment. P_{tot} accumulation under both invaded and non-invaded canopies was mostly related to root ($r_s = 0.7$, $p < 0.001$, Spearman's rank correlation) and rhizosphere mass ($r_s = 0.6$, $p < 0.01$, Spearman's rank correlation), which can also be depicted as a ratio between soil P and plant P (or OM respectively), where the soil under canopies of invaded *C. album* plants exhibited a significantly higher percentage of plant con-

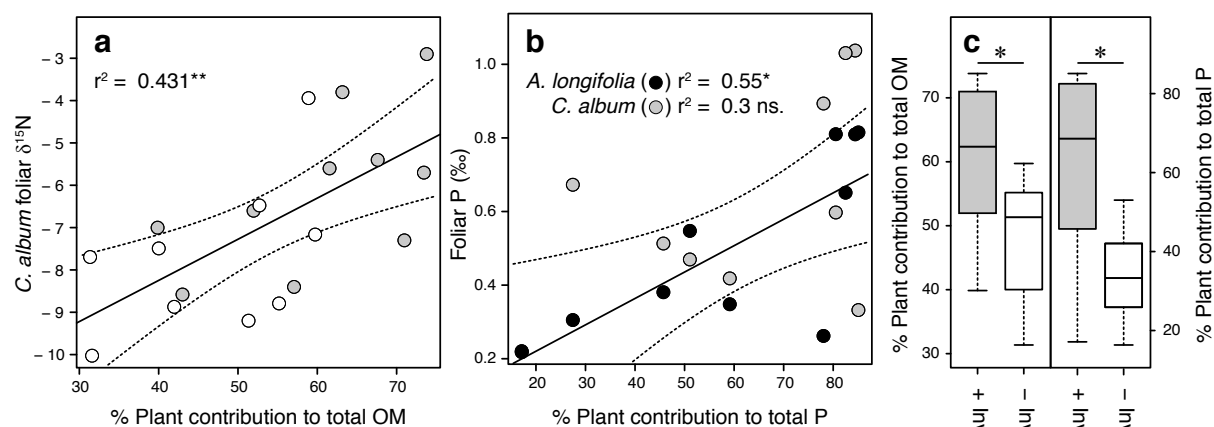


Figure 2.3 Effects of plant-derived OM and P on foliar values and the mass ratio differences in *C. album*, invaded and non-invaded by *A. longifolia*

(a) Effect of plant-derived OM (roots, rhizosphere and coarse OM), expressed as % of contribution to total organic matter, on the foliar $\delta^{15}\text{N}$ of *Corema album* plants. R^2 is result of a Pearson correlation; the dotted lines depict 95% confidence intervals. The grey spheres show invaded *C. album* plants; the white spheres show non-invaded *C. album* plants.

(b) Effect of plant phosphorus (roots, rhizosphere and coarse OM), expressed as % of contribution to total phosphorus, on the foliar P concentration of *Acacia longifolia* (black spheres) and invaded *C. album* plants (grey spheres). R^2 is a result of a Pearson correlation; the dotted lines depict 95% confidence intervals; the correlation was not significant for *C. album* plants.

(c) Mass ratios of plant OM contribution to total OM (left box) and to total P (right box) of *C. album* plants invaded (grey) and non-invaded (white) by *A. longifolia*. The asterisks highlight significant differences, * = $p < 0.05$ (Wilcoxon rank sum test, $n = 10$ for +Inv; $n = 9$ for Inv).

tribution to both total P and OM (**figure 2.3**) In contrast to the soil fractions bigger than 425 and 63 μm , the smallest soil fraction (< 63 μm) was also significantly increased underneath invaded plant canopies, both in terms of total mass and in terms of OM (both 2.3-fold). While minuscule in comparison to the other soil fractions with 0.1% contribution to total weight, OM content in this fraction doubled under invaded plants, making up 4% of the total soil OM.

Apart from changes in the overall plant biomass, also, the contribution of plant-derived material (roots, rhizosphere and coarse OM) to overall OM (plant-derived OM + OM of soil fractions) was significantly different (**figure 2.3**), which was also the case for P_{tot} . These values are percentages as they express the relative contribution to total mass in order to indicate the OM and P balance in the root-soil system. The increase of plant OM contribution to total OM was positively correlated with *C. album* foliar $\delta^{15}\text{N}$ (**figure 2.3**), which is the only variable to change significantly on foliar level after *A. longifolia* invasion (**table 2.1**). The increase of plant contribution to total P in turn was positively correlated with *A. longifolia* foliar P ratios, but not with *C. album* foliar P (**figure 2.3**).

2.4.3 Nutrient concentrations and isotopic signatures of < 63 μm soil fraction

As OM content was in general very high in this soil fraction (36%, no significant differences between species, Wilcoxon Rank Sum test, $n = 10$ for invaded *C. album*, $n = 9$ for non-invaded *C. album*, $p = 0.156$), it was possible to analyse nutrient concentrations and isotopic signatures of this material (**table 2.2**). While no significant differences between plants were found for C, N, P or stoichiometric relationship of these nutrients, both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of the OM in this fraction were significantly different underneath invaded *C. album* plants in comparison to non-invaded *C. album* plants. Similar to foliar values, invasion led to less depleted $\delta^{15}\text{N}$ values of 0.9‰ and therefore close to the atmospheric standard. In contrast, $\delta^{13}\text{C}$ values were at 27.1‰ around 0.7‰ more depleted underneath invaded *C. album* plants.

Table 2.2 Nutrient concentrations and isotopic signatures of soil fractions < 63 μm of invaded (+ Inv) and non-invaded *Corema album* (- Inv).

Significant differences ($p < 0.05$) between groups are shown in bold, Wilcoxon rank sum test, $n = 10$ for *C. album* (+ Inv) and $n = 9$ for *C. album* (- Inv).

	<i>C. album</i> (+ Inv)	<i>C. album</i> (- Inv)	p - value
$\delta^{15}\text{N}$	-0.9 (0.5)	-3 (0.7)	0.025
$\delta^{13}\text{C}$	-27.1 (0.1)	-26.4 (0.1)	0.002
% N	0.8 (0.1)	0.7 (0.1)	0.411
% C	22.3 (3.8)	16.1 (2.4)	0.278
‰ P	94.4 (17.1)	98.5 (13.3)	0.72
C:N	25.9 (2.5)	23.8 (1.3)	0.842
N:P	103.4 (16.9)	133.2 (72.2)	0.243

2.4.4 Multiple factor analysis and RV coefficients

The first two dimensions of the MFA can be seen in **figure 2.4 a**, which together explain 48% of the total variance found in the data sets. This variance is split in the first dimension, explaining 30% and second axis, explaining 18%. Variance of dimension 1 is mainly shared between OM pools (contributing with 37%) and characteristics of the < 63 μm soil fraction (contributing with 40%), while plant size expressed by volume makes up 15% and foliar-related values 9%. On the contrary, variance of dimension 2 is mainly shared between characteristics of the < 63 μm soil fraction (contributing with 39%) and foliar-related values (contributing with 48%), while OM pools contribute with 13% (**figure 2.4**). The

individual factor map (**figure 2.4 b**) shows that non-invaded *C. album* plants cluster in the lower left part, which is mainly related to less depleted $\delta^{13}\text{C}$ of the OM in the < 63 μm soil fraction, low root OM and more depleted foliar $\delta^{15}\text{N}$ values, combined with high foliar C:N ratios. RV coefficients are a multivariate version of Pearson's r^2 and calculate association strength between data matrices, in this case (**table 2.3**) the variable subsets used in the MFA and a matrix of the plant contribution to total OM and total P as depicted in **figure 2.3**. All matrices were correlated to each other, with the exception of the OM pools, which exhibited no significant association with foliage. On the contrary, plant contribution to OM and P total (termed 'mass ratios') was significantly correlated with all other matrices.

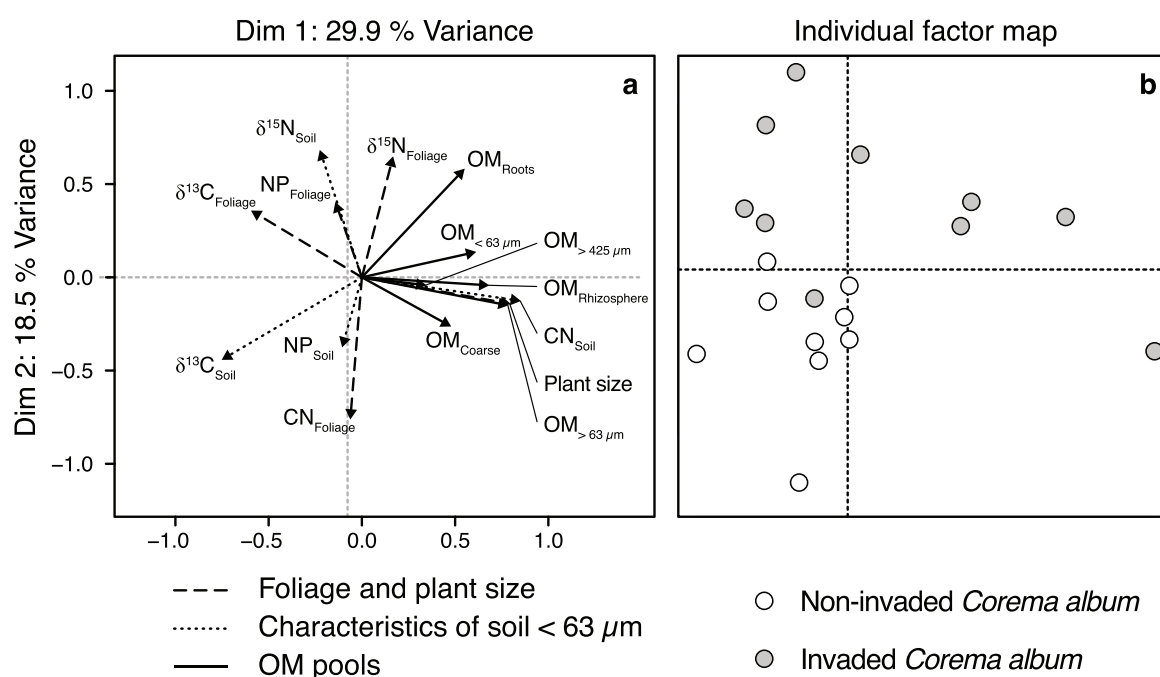


Figure 2.4 First two dimensions of a multiple factor analysis on parts of the data set from **Figure 2.2**, **Table 2.1** and **Table 2.2**. Variable types were grouped in OM pools (OM of soil fractions: > 425, > 63 and < 63 μm and of plant input: roots, rhizosphere and coarse OM), soil characteristics of the fine fraction < 63 μm (N:P ratio, C:N ratio, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) and foliar characteristics and plant size (N:P ratio, C:N ratio, $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ and volume as plant size). (a) Correlation cycle of all variables used in the multiple factor analysis; different line types indicate the different variable types used; axis denote the correlation strength (from 1 to 1). (b) Individual factor map; invaded *Corema album* plants are shown as white spheres and non-invaded *C. album* plants as grey spheres.

2.5 Discussion

As assumed in the experimental set up, *A. longifolia* invasion density in this area was low and the invasive specimens found were at an early stage of development in terms of plant volume. There was no preference of *A. longifolia* to invade *C. album* plants of a certain volume, which raises the question of how seeds arrive and establish in the canopy of *C. album*. If wind dispersal and initial soil nutrient concentration were the most important factors, canopies of larger *C. album* plants were more likely to be invaded, as there is potentially more OM accumulated (Cushman *et al.*, 2010). However, wind dispersal is unlikely for *Acacia* species, while animal dispersal by ants or birds could be a viable option for seed arrival (Wilson *et al.*, 2011), taking into account that also a wide array of animals disperse *C. album* seeds (Calvino-Cancela, 2005). While the sample size of this experiment was too low to conclude more about nursery plant preference, the lack of pattern found here emphasizes that it is important to conduct further research on this topic in order to better understand introduction pathways of *A. longifolia* seeds.

Despite this early invasion stage and the complete lack of *A. longifolia*-derived litter, there was already a clear influence of *A. longifolia* on *C. album* foliar $\delta^{15}\text{N}$, which is in line with our hypothesis about belowground plant–plant interaction. Foliar $\delta^{15}\text{N}$ is an environmental tracer for N fixation of legumes (Högberg, 1997), and even though there was no effect on foliar N concentration yet, less depleted foliar *C. album* $\delta^{15}\text{N}$ values indicate that there is an early influence of the invasive legume on its nursery plant. It has been shown that the effect on foliar $\delta^{15}\text{N}$ exceeds the effect on foliar N concentration, because changes in foliar $\delta^{15}\text{N}$ are more easily distinguishable due to the extremely low foliar $\delta^{15}\text{N}$ values of the native vegetation (Rascher *et al.*, 2012). As a legume, foliage of *A. longifolia* is more enriched in $\delta^{15}\text{N}$ and total N, which creates lower C:N ratios than those observed for the native vegetation. On the contrary, the N:P ratios were much higher in the invasive legume than in the native shrub, indicating a strong P limitation (Güsewell, 2004). These values corroborate earlier findings in the same system and could suggest an onset of systemic P depletion (Ulm *et al.*, 2017).

Table 2.3 RV coefficients of matrices used in **Figure 2.4**, as well as a matrix of mass ratios (plant contribution to total OM and P total) as shown in **Figure 2.3**.

On the bottom left corner, RV coefficients are shown; on the top right corner, p-values are shown. If p-values were smaller than 0.05, values were shown in bold.

	Foliage	Soil char. < 63 μm	OM pools	Mass ratios
Foliage		0.034	0.123	0.006
Soil char. < 63 μm	0.309		0.03	0.042
OM pools	0.262	0.338		0.004
Mass ratios	0.347	0.238	0.348	

As dunes are often co-limited in N and P, this N:P imbalance is highly detrimental to the native plants on the long term. The alleviation of N limitation by the invasive legume benefits *C. album* initially (Hellmann *et al.*, 2011); however, this comes with an exacerbation of P limitation. This P limitation could mark the shift from facilitation to resource competition, which is a natural process in the oligotrophic dunes (Martínez and García-Franco, 2008), where resource use efficiency is crucial for successful plant invasion (Funk and Vitousek, 2007). Indeed, *Acacia* species are known to be very P use efficient (Inagaki and Tange, 2014), which, together with their ability to fix atmospheric N, gives them a considerable advantage in oligotrophic ecosystems. The native vegetation, in contrast, is highly adapted to the low resource environment and has a more conservative resource use, which might impede them from benefiting as much from additional N as the invasive legume (Hellmann *et al.*, 2011). Belowground, invaded *C. album* shrubs did not differ from non-invaded *C. album* shrubs in terms of levels of coarse OM but exhibited increased root and rhizosphere biomass, which were the main sources for OM differences in invaded *C. album* plants. The strong increase in belowground OM was not proportional to the aboveground increase in biomass, indicating that at an early stage, belowground parameters might be more sensitive to invasion in contrast to impacts aboveground, which supports observations from other invasive species that early invasions might develop in a non-linear fashion (Elgersma and Ehrenfeld, 2011). It has been shown that the longer *A. longifolia* invasion persists, the more detrimental its belowground impacts are (March-

ante *et al.*, 2008), and the data presented here add a further point in this timeline. While P and OM increase could benefit both plants growing together and the OM shift to the biotic compartment is positively related with the effect on *C. album* foliar $\delta^{15}\text{N}$, *A. longifolia* foliar P indicates that it takes more advantage of the P accumulation in the biotic compartment. This might be due to its increased root mass but could also be due to the rhizosphere and the microbial processes therein, as this compartment is crucial in P acquisition, for example through mycorrhizal fungi (Philippot *et al.*, 2013). Changes in microbial community structure and soil conditioning by *A. longifolia* could be highly detrimental to native plant interactions, as they might lead to ‘invasional meltdown’, which describes the co-introduction of non-native symbiotic nitrogen-fixing bacteria (Rodríguez-Echeverría, 2010). It was proposed that the introduction of new soil biota might potentially disrupt native mutualisms and create negative feedback loops; however, it seems that similar to *C. album* also native legumes, such as *Ulex europaeus* and *Cytisus grandiflorus*, benefit from the soil alterations by *A. longifolia* (Rodríguez-Echeverría *et al.*, 2009). On the other hand, positive interactions of these native legumes with soil altered by *A. longifolia* could be a chance for native species restoration, as both species were found to be suitable for revegetation of sites formerly invaded by *A. longifolia* (Rodríguez-Echeverría *et al.*, 2009).

Apart from differences at plant level, soil under invaded *C. album* canopies exhibited an increase in the silt-clay fraction ($< 63 \mu\text{m}$). This fraction is mainly related to microbially stabilized biomass (Grandy and Neff, 2008),

and its increase might be of crucial importance for further soil OM aggregation (Six *et al.*, 2002), which is also evident from the high N, C and P contents in this fraction. Similar to foliar nutrient values, there were no differences in total N, C or P; however, $\delta^{15}\text{N}$ values and $\delta^{13}\text{C}$ were already altered in invaded canopies, which is a further example of the usefulness in using stable isotope signatures as early warning signs of ecological change, such as plant invasion (Williams *et al.*, 2007). Less depleted soil $\delta^{15}\text{N}$ was expected due to nitrogen fixation by *A. longifolia*, and the values observed here were similar to root $\delta^{15}\text{N}$ values reported in earlier works (Ulm *et al.*, 2017), thus highlighting them as a potential source.

Interestingly, also the $\delta^{13}\text{C}$ values of this compartment were significantly depleted underneath invaded *C. album* plants. As this compartment must necessarily be relatively recent due to small *A. longifolia* plant volume, $\delta^{13}\text{C}$ depletion due to age effects, such as leaching, is unlikely. More probable is the accumulation of lignin and aliphatic biopolymers in this fraction (Creamer *et al.*, 2011) and a higher ratio of fungal/bacterial biomass (Kohl *et al.*, 2015). An increase in fungal biomass in turn could have important long-term effects for cross facilitation for other invasive *Acacia* species, as they require mycorrhizal symbionts for P upkeep (Nuñez and Dickie, 2014). The difference in $\delta^{13}\text{C}$ was also highlighted in the MFA as an important factor distinguishing invaded from non-invaded *C. album* canopies, the latter also being associated with low root OM and more depleted foliar $\delta^{15}\text{N}$ values. Interestingly, the clustering of non-invaded *C. album* plant canopies was visible on both the first and the second axes, which were related

to the silt-clay fraction with 40%, stressing this compartment a major factor for their characterization. Also, looking at the relationships between the variable types, it becomes clear that OM pools alone are not related to the foliar changes observed, while both the silt-clay fraction and the soil–plant biomass ratios are correlating well with all other variables measured. As these belowground changes often go unnoticed but precede the aboveground changes observed later in these systems, these data add a further argument for the high ‘invasion debt’, a term describing the delay in invasion impact along time, of *Acacia* species (Richardson *et al.*, 2015).

Summarized, it can be stated that *A. longifolia* exhibits an impact on *C. album* foliar $\delta^{15}\text{N}$ already at an early invasion phase, accompanied by strong changes belowground, which, at least at this stage, are not related to foliar litter or coarse OM. At soil level, the differences were mainly associated with the silt-clay fraction, which was more abundant with the presence of the invader and constitutes an important pool of OM. We were able to detect differences in both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ of the OM found in this compartment, underlining its putative invasive origin and highlighting the importance of further research on this soil fraction. Furthermore, we found that roots and rhizosphere play fundamental roles early on in invasion and that the ratio of OM and P in the biotic compartment against the abiotic (soil) compartment is an indicator for differences in soil parameters in invaded and non-invaded *C. album* canopies. As these differences were found in *C. album* plants without aboveground *A. longifolia* biomass accumulation, it is imperative for conservation to tackle *A. longi-*

folia invasion early on, before soil changes become irreversible. In conclusion, the work presented here adds important evidence to understand the first belowground alterations occurring in the process of *A. longifolia* invasion, which might help to mitigate damages done by this prolific invasive species, which continues to degrade many ecosystems worldwide.

Acknowledgements

We want to thank the Estabelecimento Prisional de Pinheiro da Cruz for allowing the establishment of the plots and Maria Carolina Nunes Alves da Silva and Gláucia Soares Tolentino for the help with fieldwork as well as Catarina Gomes and Mariana Barreira for lab assistance. This work was funded by the PhD grant PD/BD/106061/2015 from FCT (Fundação para a Ciência e a Tecnologia) and the project FP7-PEOPLE- 2010-IRSES from the Seventh Framework Programme.

2.6 References

- Armas, C. and Pugnaire, F.I., 2005.** Plant interactions govern population dynamics in a semi-arid plant community. *Journal of Ecology*, 93(5), pp.978-989.
- Borcard, D., Gillet, F. and Legendre P., 2011.** Canonical ordination. In: *Numerical ecology with R*. Springer: New York; pp.153–224.
- Calvino-Cancela, M., 2005.** Fruit consumers and seed dispersers of the rare shrub *Corema album*, Empetraceae, in coastal sand dunes. *Revue d'Écologie (Terre Vie)* 60: pp.97–106.
- Charbonneau, B.R., Wootton, L.S., Wnek, J.P., Langley, J.A. and Posner, M.A., 2017.** A species effect on storm erosion: Invasive sedge stabilized dunes more than native grass during Hurricane Sandy. *Journal of Applied Ecology*, 54(5), pp.1385-1394.
- Creamer, C.A., Filley, T.R., Boutton, T.W., Oleynik, S. and Kantola, I.B., 2011.** Controls on soil carbon accumulation during woody plant encroachment: Evidence from physical fractionation, soil respiration, and $\delta^{13}\text{C}$ of respired CO_2 . *Soil Biology and Biochemistry*, 43(8), pp.1678-1687.
- Cushman, J.H., Waller, J.C. and Hoak, D.R., 2010.** Shrubs as ecosystem engineers in a coastal dune: influences on plant populations, communities and ecosystems. *Journal of Vegetation Science*, 21(5), pp.821-831.
- D'Angelo, E., Crutchfield, J. and Vandiviere, M., 2001.** Rapid, sensitive, microscale determination of phosphate in water and soil. *Journal of Environmental Quality*, 30(6), pp.2206-2209.
- Doody, J.P., 2013.** Alien plant invasion. In: *Sand dune conservation, management and restoration*. Springer: Netherlands, pp.177–199.
- Elgersma, K.J. and Ehrenfeld, J.G., 2011.** Linear and non-linear impacts of a non-native plant invasion on soil microbial community structure and function. *Biological Invasions*, 13(3), pp.757-768.
- Fei, S., Phillips, J. and Shouse, M., 2014.** Biogeomorphic impacts of invasive species. *Annual Review of Ecology, Evolution, and Systematics*, 45, pp.69–87.
- Funk, J.L. and Vitousek, P.M., 2007.** Resource-use efficiency and plant invasion in low-resource systems. *Nature*, 446(7139), p.1079.
- Grandy, A.S. and Neff, J.C., 2008.** Molecular C dynamics downstream: the biochemical decomposition sequence and its impact on soil organic matter structure and function. *Science of the Total Environment*, 404(2-3), pp.297-307.
- Güsewell, S., 2004.** N: P ratios in terrestrial plants: variation and functional significance. *New Phytologist*, 164(2), pp.243-266.
- Hellmann, C., Sutter, R., Rascher, K.G., Máguas, C., Correia, O. and Werner, C., 2011.** Impact of an exotic N_2 -fixing *Acacia* on composition and N status of a native Mediterranean community. *Acta Oecologica*, 37(1), pp.43-50.
- Hellmann, C., Werner, C. and Oldeland, J., 2016.** A spatially explicit dual-isotope approach to map regions of plant-plant interaction after exotic plant invasion. *PloS one*, 11(7), p.e0159403.
- Hogberg, P., 1997.** ^{15}N natural abundance in soil–plant systems. *Tansley Review No. 95*. *New Phytologist*, 137, pp.179-203.
- Inagaki, M. and Tange, T., 2014.** Nutrient accumulation in aboveground biomass of planted tropical trees: a meta-analysis. *Soil Science and Plant Nutrition*, 60(4), pp.598-608.
- Jones, C.G., Lawton, J.H. and Shachak, M., 1994.** Organisms as ecosystem engineers. In: *Ecosystem management*. Springer, New York, pp.130-147.
- Kohl, L., Laganière, J., Edwards, K.A., Billings, S.A., Morrill, P.L., Van Biesen, G. and Ziegler, S.E., 2015.** Distinct fungal and bacterial $\delta^{13}\text{C}$ signatures as potential drivers of increasing $\delta^{13}\text{C}$ of soil organic matter with depth. *Biogeochemistry*, 124(1-3), pp.13-26.
- Ludwig, J.A., Reynolds, J.F. and Whitson, P.D., 1975.** Size-biomass relationships of several Chihuahuan desert shrubs. *American Midland Naturalist*, pp.451-461.
- Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2008.** Short-and long-term impacts of *Acacia longifolia* invasion on the belowground processes of a Mediterranean coastal dune ecosystem. *Applied Soil Ecology*, 40(2), pp.210-217.
- Marchante, H., Marchante, E., Freitas, H. and Hoffmann, J.H., 2015.** Temporal changes in the impacts on plant communities of an invasive alien tree, *Acacia longifolia*. *Plant Ecology*, 216(11), pp.1481-1498.
- Martínez, M.L. and García-Franco, J.G., 2008.** Plant–plant interactions in coastal dunes. In: *Coastal Dunes*. Springer: Berlin Heidelberg, pp.205–220.
- McClaran, M.P., Moore-Kucera, J., Martens, D.A., van Haren, J. and Marsh, S.E., 2008.** Soil carbon and nitrogen in relation to shrub size and death in a semi-arid grassland. *Geoderma*, 145(1-2), pp.60-68.
- Morris, T.L., Esler, K.J., Barger, N.N., Jacobs, S.M. and Cramer, M.D., 2011.** Ecophysiological traits associated with the competitive ability of invasive Australian acacias. *Diversity and Distributions*, 17(5), pp.898-910.

- Muhamed, H., Touzard, B., Le Bagousse-Pinguet, Y. and Michalet, R., 2013.** The role of biotic interactions for the early establishment of oak seedlings in coastal dune forest communities. *Forest Ecology and Management*, 297, pp.67-74.
- Núñez, M.A. and Dickie, I.A., 2014.** Invasive below-ground mutualists of woody plants. *Biological Invasions*, 16(3), pp.645-661.
- Peperkorn, R., 2005.** Key factors of invasiveness of *Acacia longifolia* – experimental comparison with native species from Mediterranean sand ecosystem. PhD-Thesis, University of Bielefeld.
- Philippot, L., Raaijmakers, J.M., Lemanceau, P. and Van Der Putten, W.H., 2013.** Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, 11(11), p.789.
- R Core Team., 2016.** R: a Language and Environment for Statistical Computing. R. Foundation for Statistical Computing, Vienna, Austria.
- Rascher, K.G., Hellmann, C., Máguas, C. and Werner, C., 2012.** Community scale ^{15}N isoscapes: tracing the spatial impact of an exotic N_2 -fixing invader. *Ecology Letters*, 15(5), pp.484-491.
- Richardson, D.M., Hui, C., Nunez, M.A. and Pauchard, A., 2014.** Tree invasions: patterns, processes, challenges and opportunities. *Biological Invasions*, 16(3), pp.473-481.
- Richardson, D.M., Le Roux, J.J. and Wilson, J.R., 2015.** Australian acacias as invasive species: lessons to be learnt from regions with long planting histories. *Southern Forests: a Journal of Forest Science*, 77(1), pp.31-39.
- Rodríguez-Echeverría, S., 2010.** Rhizobial hitchhikers from Down Under: Invasional meltdown in a plant–bacteria mutualism? *Journal of Biogeography*, 37(8), pp.1611-1622.
- Rodríguez-Echeverría, S., Crisóstomo, J.A., Nabais, C. and Freitas, H., 2009.** Belowground mutualists and the invasive ability of *Acacia longifolia* in coastal dunes of Portugal. *Biological Invasions*, 11(3), pp.651-661.
- Rundel, P.W., Dickie, I.A. and Richardson, D.M., 2014.** Tree invasions into treeless areas: mechanisms and ecosystem processes. *Biological Invasions*, 16(3), pp.663-675.
- Schwarz, C., Ysebaert, T., Vandenbruwaene, W., Temmerman, S., Zhang, L. and Herman, P.M., 2016.** On the potential of plant species invasion influencing bio-geomorphologic landscape formation in salt marshes. *Earth Surface Processes and Landforms*, 41(14), pp.2047-2057.
- Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M. and Pyšek, P., 2013.** Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution*, 28(1), pp.58-66.
- Six, J., Conant, R.T., Paul, E.A. and Paustian, K., 2002.** Stabilization mechanisms of soil organic matter: implications for C-saturation of soils. *Plant and Soil*, 241(2), pp.155-176.
- Ulm, F., Hellmann, C., Cruz, C. and Máguas, C., 2017.** N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume. *Oikos*, 126(2).
- Van der Putten, W.H., Bardgett, R.D., Bever, J.D., Bezemer, T.M., Casper, B.B., Fukami, T., Kardol, P., Klironomos, J.N., Kulmatiski, A., Schweitzer, J.A. and Suding, K.N., 2013.** Plant–soil feedbacks: the past, the present and future challenges. *Journal of Ecology*, 101(2), pp.265-276.
- Werner, C., Zumkier, U., Beyschlag, W. and Máguas, C., 2010.** High competitiveness of a resource demanding invasive acacia under low resource supply. *Plant Ecology*, 206(1), pp.83-96.
- Williams, D.G., Evans, R.D., West, J.B. and Ehleringer, J.R., 2007.** Applications of stable isotope measurements for early-warning detection of ecological change. *Terrestrial Ecology*, 1, pp.383-398.
- Wilson, J.R., Gairifo, C., Gibson, M.R., Arianoutsou, M., Bakar, B.B., Baret, S., Celesti-Grapow, L., DiTomaso, J.M., Dufour-Dror, J.M., Kueffer, C. and Kull, C.A., 2011.** Risk assessment, eradication, and biological control: global efforts to limit Australian acacia invasions. *Diversity and Distributions*, 17(5), pp.1030-1046.

Chapter 3

N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume

Ulm, F., Hellmann, C., Cruz, C. and Máguas, C., 2017. N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume. *Oikos*, 126(2).

3.1 Abstract

Oligotrophic ecosystems, previously considered to be more resilient to invasive plants, are now recognised to be highly vulnerable to invasions. In these systems, woody legumes show belowground ecosystem engineering characteristics that enable invasion, however, the underlying processes are not well understood. Using a Portuguese primary dune ecosystem as an oligotrophic model system, belowground biomass pools, turnover rates and stoichiometry of a native (*Stauracanthus spectabilis*) and an invasive legume (*Acacia longifolia*) were compared and related to changes in the foliage of the surrounding native (*Corema album*) vegetation.

We hypothesized that the invasive legume requires less phosphorus per unit of biomass produced and exhibits an enhanced nutrient turnover compared to the native vegetation, which could drive invasion by inducing a systemic N/P imbalance.

Compared with the native legumes, *A. longifolia* plants had larger canopies, higher SOM levels and lower tissue P concentrations. These attributes were strongly related to legume influence as measured by increased foliar N content and less depleted $\delta^{15}\text{N}$ signatures in the surrounding *C. album* vegetation. Furthermore, higher root N concentration and increased nutrient turnover in the rhizosphere of the invader were associated with depleted foliar P in *C. album*.

Our results emphasize that while *A. longifolia* itself maintains an efficient phosphorus use in biomass production, at the same time it exerts a strong impact on the N/P balance of the native system. Moreover, this study highlights the engineering of a belowground structure of roots and rhizosphere as a crucial driver for invasion, due to its central role in nutrient turnover. These findings provide new evidence that, under nutrient-limited conditions, considering co-limitation and nutrient cycling in oligotrophic systems is essential to understand the engineering character of invasive woody legumes.

3.2 Introduction

Invasive plant species are contributing significantly to human-induced global change due to their strong impacts on biodiversity and ecosystem functioning (Simberloff *et al.* 2013). However, effects of invasion on soil nutrient cycling have long been overlooked and especially comparison studies of native versus invasive species rarely consider belowground plant traits (Smith *et al.* 2014). Thus, soil properties and nutrient cycling have become major issues in the research of plant invasion (Hulme *et al.* 2013) with particularly the role of nitrogen (N) and phosphorus (P) relationships between plant and soil requiring further research (Sardans and Peñuelas 2012).

Belowground processes play a crucial role for the invasion of woody plants (Nuñez and Dickie 2014), which is a group of invasive plants receiving increasing attention (Richardson and Rejmánek 2011). Woody legumes in particular can fundamentally alter ecosystem function, e.g. by facilitating succession of tree species in their litter and rhizosphere zone (Bellingham *et al.* 2001) or by exerting competitive pressure under non-favourable conditions (Watt *et al.* 2003). While invasive woody legume species are known to be able to provide N to the ecosystem by symbiotic nitrogen fixation (Augusto *et al.* 2005), this also leads to P limitation in nutrient poor ecosystems (Augusto *et al.* 2013). However, even though P turnover is a crucial component of ecosystem functions, its relevance for plant invasions has rarely been addressed (Ehrenfeld 2010).

Acacia longifolia is a globally “very widespread” (Richardson and Rejmánek 2011) invasive legume and is also termed an “ecosystem engineer” sensu Badano *et al.* (2010). It is also one of the most influential invaders in dune systems in several countries worldwide (Marchante *et al.* 2008), which are both ecosystems with high conservational value and excellent model systems for invasion biology, as they allow for the observation of engineering effects of invasive species on ecological timescales due to their transitional nature and intermediate energy balance (Fei *et al.* 2014). For example, *A. longifolia* fundamentally transforms the oligotrophic dune systems by promoting monospecific plant communities (Hellmann *et al.* 2011), profoundly altering edaphic conditions, increasing soil organic matter (OM) levels (Marchante *et al.* 2008, Hellmann *et al.* 2011) and creating a positive feedback loop between plant and soil (Marchante *et al.* 2008).

The success of the *Acacia* genus has been mainly ascribed to high nutrient use efficiency, its ability fix N, elevated growth rates, larger size and a bigger investment in root mass, both in terms of deep roots and shallow root networks (Morris *et al.* 2011, Funk 2013). Roots in turn have direct effects on the adjacent rhizosphere, which is increasingly recognized for its role in ecological engineering and exotic plant invasions (Philippot *et al.* 2013). Also, litter mass plays an important role in *Acacia spp.* invasion, due to their high leaf turnover rates as well as extensive leaf shedding in stress conditions (Rascher *et al.* 2012), which in the case of *A. longifolia* leads to the

accumulation of a thick litter layer underneath the canopy (Marchante *et al.* 2008). Not only OM pool sizes, e.g. root, rhizosphere and litter mass, but also flux rates, such as litter and fine root decomposition, increase in invaded ecosystems (Liao *et al.* 2008).

Biomass decomposition rates of plant tissues strongly depend on the constrained stoichiometry between carbon (C), N and P requirements of the microorganisms degrading them (Manzoni *et al.* 2010). While stoichiometric ratios of plant tissues can shed light on nutrient limitation (Güsewell 2004), extracellular enzyme activities (EEA) are useful measures for potential nutrient turnover (Sinsabaugh *et al.* 2009). The enzymatic turnover of N and P is tightly coupled to each other as well as to C release (Manzoni *et al.* 2010) and can be used as a functional measure of nutrient and energy flow (Sinsabaugh *et al.* 2009). Among the most widely assessed activities are those of β -1,4-glucosidase (Glc), β -1,4-N-acetylglucosaminidase (Nag) and acid phosphatase (Ap) (Sinsabaugh *et al.* 2009), which are of crucial importance to energy, N and P turnover, respectively.

Invasive and native species have effects on soil nutrient cycling that can be estimated by measuring variables of the surrounding plant community, for example leaf stoichiometry (Sardans and Peñuelas 2012) or ecological tracers such as foliar $\delta^{15}\text{N}$ (Hellmann *et al.* 2011, Rascher *et al.* 2012). Natural abundance $\delta^{15}\text{N}$ measurements can be used to quantify the proportion of N derived from atmospheric N_2 fixation by legumes in plant–soil systems (Unkovich 2013) and foliar $\delta^{15}\text{N}$ is a good proxy for soil $\delta^{15}\text{N}$, especially under low N availability (Craine *et al.* 2009). This approach

was successfully used in the oligotrophic Portuguese primary dunes, utilizing foliar $\delta^{15}\text{N}$ of the non-leguminous native shrub *Corema album* (Hellmann *et al.* 2011, Rascher *et al.* 2012). Similar to other ericoid mycorrhizal plants (Craine *et al.* 2009), *C. album* exhibits very depleted foliar $\delta^{15}\text{N}$ values without legume influence, which, together with its high abundance in this system, suggest it to be a good monitoring plant for legume influence (Rascher *et al.* 2012). As the invasive *A. longifolia* and *Stauracanthus spectabilis*, a native, sclerophyllous, leguminous shrub are the only legumes in this system and the two species co-occur with *C. album* in these very oligotrophic primary dunes with no further sources of OM input, this situation creates a model system that is ideally suited to quantify the impact of *A. longifolia* invasion (Rascher *et al.* 2012).

It has been shown that *C. album* plants in close proximity of *A. longifolia* exhibit enhanced growth rates, higher foliar N contents and less depleted $\delta^{15}\text{N}$ values (Hellmann *et al.* 2011), these being effects that were not found in the proximity of the native *S. spectabilis*. Because *A. longifolia* will ultimately dominate the invaded ecosystem, these findings are somewhat paradox as they indicate that *C. album* plants seem to benefit from the N supply of the invasive legume. However, woody legumes, like invasive *Acacia* species, have a high N and P demand (Augusto *et al.* 2013, Mortimer *et al.* 2013) and as P is mainly released by rock weathering, plant growth can further sink-deplete the residing P pool (Vitousek *et al.* 2010). Following this reasoning, legume influence on surrounding *C. album* plants should cause a systemic increase in N availability accompanied by P depletion. It was therefore hypoth-

esized that *A. longifolia*, contrary to the native legume, creates an N/P imbalance by inducing belowground processes that enhance nutrient cycling and increase OM underneath its own canopy. To test this hypothesis, we quantified the mass and stoichiometry of major below-ground OM pools as well as their potential turnover rates for both legumes (native and invasive) and related these variables to changes in the native *C. album* foliar N, $\delta^{15}\text{N}$ and total P of the surrounding vegetation.

3.3 Materials and Methods

3.3.1 Study site and species description

Sampling took place from 21 to 23 May 2012 in the coastal sand dune ecosystem of Pinheiro da Cruz, Portugal (38°15.2'N, 8°45.8'W). The site is located in the primary dunes, which are characterized by poor arenosols (FAO classification) and by an open vegetation structure (Rascher *et al.* 2012). The vegetation is dominated by *Corema album* (Ericaceae), a shrub endemic to the Iberian Peninsula and two nitrogen fixing leguminous species, the native *Stauracanthus genistoides*, subspecies *spectabilis* which is a sclerophyllous leguminous shrub (Barradas *et al.* 1999) and the invasive non-native leguminous tree *Acacia longifolia* (Fabaceae).

3.3.2 Plant sampling design and in situ measurements

Samples and measurements were taken underneath five *S. spectabilis* and five *A. longifolia* plants. Plant canopy sizes were estimated as ellipses from 2 radii. Underneath each plant, three sampling points were randomly selected and litter height was measured on 12 random spots around each sampling point. Starting from the sampling points, mature, sunlit leaves from 15 *C. album* plants were sampled along three transects (five *C. album* plants per transect) with a mean distance 6 standard error of 1.35 ± 0.16 m between each plant (**figure 3.1**) and transect lengths of 6.33 ± 1.2 m for *S. spectabilis* and 9.95 ± 0.81 m for *A. longifolia*.

3.3.3 Soil collection and preparation

At each sampling point, soil was collected with a metal tube of 8.5 cm in diameter and 19.5 cm in height. Subsequently, samples were kept at 4°C in airtight plastic bags until analysis. Bulk samples were separated into four fractions (sand, rhizosphere, roots and litter). The top organic layer of the sample up until the onset of the sand layer was removed and termed litter. The sand fraction was removed from the bulk-agglomerated rhizosphere/root in the core and then sieved through a 1-mm sieve. Rhizosphere was separated from roots by gently shaking the adhered particles off the roots and subsequently treating them similar to the soil. Parts of all fractions were oven-dried at 60°C until constant weight and 500 mg were ashed in a muffle furnace (600°C, 24 h) to determine organic matter by the loss on ignition method.

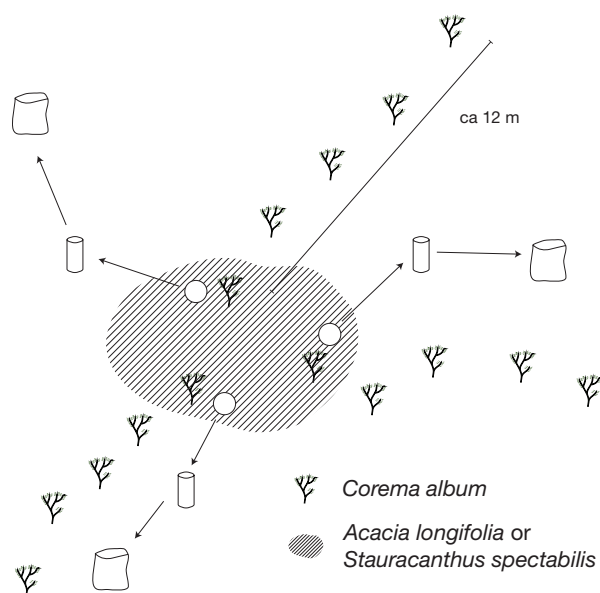


Figure 3.1 Schematic of the experimental setup. For each legume, 5 plant canopies were chosen and then 3 transects laid out, as depicted in the schematic. Along these transects, foliage of *C. album* was sampled, with 5 plants sampled per transect. Thus, the sampling design was nested, with transect nested in plant ($n = 5$ per legume).

Soil nutrients and pH were analysed in 1:10 (m/v) soil extracts done from both rhizosphere and sand samples in ultrapure water with 30 min extraction time on a shaker at room temperature. To obtain a clear extract, the resulting soil-water suspension was centrifuged (5000 g, 4°C, 15 min) and finally filtered through sterile medical gauze with a pore size of 500 µm. pH was subsequently analysed in the extract with a glass electrode (pH/mV meter).

3.3.4 Colorimetric assays

Concentrations of NH_4^+ , NO_3^- and soluble inorganic P of soil extracts was quantified by colorimetric assays. To determine total phosphorus of soil and organic matter, complete samples were ignited, followed by acid-extraction (HCl, 1 M) and inorganic phosphorus determination. All colorimetric assays were done using microscale methods, executed in 250 µl 96-well flat bottom microtiter plates and analysed in a microplate reader. Reaction vessels were acid-washed, bleach-washed and thoroughly rinsed with distilled water before usage. For each single assay a separate triplicate calibration curve was produced with the respective salts (KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, KNO_3) as serial dilutions in ultrapure water. Soluble inorganic phosphorus was analysed using a malachite-green based method described by D'Angelo *et al.* (2001). Organic phosphorous in soil and rhizosphere was subsequently calculated from inorganic and total phosphorous. NH_4^+ was analysed using a modified Berthelot reaction (Cruz and Martins-Loução 2000) and NO_3^- using the VCl_3 /Griess method as described by Hood-Nowotny *et al.* (2010).

3.3.5 Fluorometric enzyme activity profiling

The procedures, solutions and incubation times were followed as described by Pritsch *et al.* (2011). Root tips were carefully separated from the attached sand, rinsed in tap water and ca 2 mm sized root parts were transferred into 96-well filter plates (96-filter plate with 30 - 40 µm mesh size) where they stayed for the whole assay procedure. The enzyme tests were performed using fluorescent substrate analogues labelled with methylumbelliferone (MU): MU-xyloside, MU-glucuronide, MU-cellobiohydrofuran, MU-N-acetylglucosamine, MU-β-glucoside and MU-phosphate and fluorescence measured at 364 nm excitation and 450 nm emission in a fluorescence microplate reader. The values obtained were calibrated against a standard curve and related to the total area of root tips by scanning the root parts and analysing the pictures with Photoshop for surface estimation.

Litter was crushed and homogenized into small pieces using a pair of clean scissors; 20 mg was weighed on a microscale and then transferred to the wells. Rhizosphere samples were mixed thoroughly prior to weighing and also 20 mg was transferred to the wells. The values obtained were calibrated against a standard curve and related to the organic matter weight.

3.3.6 Elemental analysis and isotopic ratios

Dry organic matter samples of foliage, roots and litter were ground to a fine powder in a ball mill. 5 ± 0.2 mg of the powder was weighed into tin capsules and $^{15}\text{N}/^{14}\text{N}$ ratios in the samples were determined on a continuous flow stable isotope ratio mass spectrometer coupled to an elemental analyser for online sample preparation by Dumas-combustion. The standards used were IAEA-N₁ and USGS-35 for nitrogen isotope ratio, $\delta^{15}\text{N}$ results were referred to air. Precision of the isotope ratio analysis, calculated using values from six to nine replicates of laboratory standard material interspersed among samples in every batch analysis, was $\leq 0.2\text{‰}$.

3.3.7 Statistical analysis

If not stated otherwise, all tests were performed with the package stats using version R 3.2.4 (R Core Team, 2016). Comparisons between biomass pools and specific extracellular enzyme activities of rhizosphere and litter of both legumes were performed using Wilcoxon rank sum tests. This test was also used to compare differences between legume influence on *C. album* foliar % N, P and $\delta^{15}\text{N}$. To test for significant differences between soil and rhizosphere of both legume species, pairwise Wilcoxon rank sum tests with Holm adjustment were used.

In order to find the spatial threshold of legume influence, the relationship between *C. album* $\delta^{15}\text{N}$ and N content, respectively, with the distance to the legume canopy was investigated. Since $\delta^{15}\text{N}$ and N content both increased in the vicinity to the legume canopy, a breakpoint was expected reflecting the threshold between

the influenced range and the background value. To estimate this breakpoint, segmented regressions were fitted using the function *segmented.lme()* (Muggeo *et al.* 2014, modified by V. Muggeo to handle nested random effects). Distance to the legume canopy was included as fixed effect and legume and transect were included as random effects, with transect nested in legume (**figure 3.1**). The models were fitted by log-likelihood maximisation. Model terms, including breakpoints of all models, were tested for significance with the Wald-test using *anova.lme()*. After breakpoint estimation, plants were grouped into plants growing in the canopy (IN) and plants growing outside (OUT) of breakpoint range. Legume influence was then defined as the difference between IN and OUT (IN - OUT) for $\delta^{15}\text{N}$ and the % of change ((IN - OUT)/IN) for total foliar N. Assumptions for the linear regression between % of change in *C. album* foliar total N and foliar total P (‰) were checked by the Breusch–Pagan test against homoscedasticity and the residuals checked for normality using the Shapiro–Wilk normality test. These assumptions were also verified for all mentioned correlations in the text.

Variables significant to explain legume influence were identified based on partial least squares (PLS) regression and backward selection of significant variables using the *autopls()* function from the autopls package (Schmidtlein *et al.* 2012), which selects variables on the basis of variable importance in the projection (VIP) and significance for prediction. The variables used for the selection procedure were: plant size, ΣC litter, Ap litter, Nag litter, ΣC roots, Ap roots, Nag roots, ΣC rhizosphere, Ap rhizosphere, Nag rhizosphere,

litter mass, root mass, rhizosphere mass, soil OM, rhizosphere OM, soil organic P, rhizosphere organic P, total N litter, total P litter, total N roots, total P roots. The PLSR approach employs linear regression to project observed and predicted variables to a new space, thus creating components that are both explaining variance within the set of predictor variables as well as the variance of the observed variable. The procedure is creating a prediction model using an optimization procedure based on variable significance and is internally validated by leave-one-out cross-validation. Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.gc735>

3.4 Results

3.4.1 Pool sizes and stoichiometry

Comparison of the native and the invasive legumes, *Stauracanthus spectabilis* and *Acacia longifolia*, revealed differences in size and belowground biomass pools (**table 3.1**), with

the canopy of the invasive *A. longifolia* being 12 fold larger than the canopy of the native *S. spectabilis*. The invasive also exhibited a significantly larger root mass and a slightly larger rhizosphere. In terms of soil parameters, both

Table 3.1 Characterization of the biomass pools associated with the invasive *Acacia longifolia* and the native *Stauracanthus spectabilis* legumes.

Three biomass pools were considered: leaf, litter and root. Rhizosphere/soil ratio was calculated as rhizospheric soil relative to the bulk soil sample (w/w). Values are means (n = 5) with standard errors given in brackets, rows in bold show significant differences between groups (p < 0.05, Wilcoxon test).

		<i>Acacia longifolia</i>	<i>Stauracanthus spectabilis</i>	p - value
Plant size	Canopy (m²)	75.0 (18.7)	6.5 (0.4)	0.008
Biomass pools	Litter (kg m ⁻²)	3.3 (0.4)	3.6 (0.7)	1
	Root (kg m⁻²)	2.9 (0.9)	0.9 (0.2)	0.032
	Rhizosphere (kg m ⁻²)	79.2 (27.1)	15.5 (6.7)	0.056
	Rhizosphere/Soil ratio (w/w)	0.32 (0.11)	0.07 (0.03)	0.056
Leaf biomass	δ¹⁵N	-2.6 (0.2)	-0.6 (0.2)	0.008
	% N	1.9 (0.1)	1.8 (0.1)	0.421
	% C	47.5 (1.2)	47.8 (1.3)	0.841
	‰ P	0.3 (<0.1)	0.9 (<0.1)	0.008
	C:N	25.4 (1.7)	27.3 (1.1)	0.548
	N:P	59.6 (7.8)	19.9 (0.8)	0.008
	C:P	1512.8 (237.7)	540.8 (26.8)	0.008
Litter biomass	δ ¹⁵ N	-1.7 (0.4)	-1.6 (0.2)	1
	% N	1.4 (0.2)	1.5 (0.1)	0.548
	% C	42.5 (1.9)	43.5 (0.9)	0.421
	‰ P	0.3 (0.1)	0.4 (<0.1)	0.222
	C:N	31.9 (2.4)	29.4 (1.7)	0.31
	N:P	85.6 (24.8)	43.9 (3.7)	0.222
	C:P	2429.5 (566.4)	1295.6 (157.4)	0.095
	Thickness (cm)	14.6 (2.8)	28.8 (3.3)	0.016
Root biomass	δ¹⁵N	-0.5 (0.1)	-1.9 (0.3)	0.008
	% N	1.2 (0.2)	0.7 (0.1)	0.056
	% C	36.6 (1.9)	39.1 (2.3)	0.548
	‰ P	0.2 (0)	0.4 (0.1)	0.69
	C:N	33.7 (3.5)	74.8 (18.4)	0.032
	N:P	83.7 (29.3)	37.3 (12.5)	0.151
	C:P	2104.5 (667.6)	2136.2 (569.9)	1

Table 3.2 Characteristics of the soil and the rhizosphere compartments of the two studied legumes, the invasive *Acacia longifolia* and the native *Stauracanthus spectabilis*. Values are means (n = 5) with standard errors given in brackets. Different letters indicate significant differences (p < 0.05, pairwise Wilcoxon test with Holm correction).

	Species	% Organic matter	pH	µg g ⁻¹ Soluble P	µg g ⁻¹ Organic P	µg g ⁻¹ Nitrate	µg g ⁻¹ Ammonium
Soil	<i>A. longifolia</i>	0.4 (0) ^{ab}	5.6 (0.1) ^a	0.3 (0.1) ^a	5 (1.0) ^a	2.6 (0.4) ^a	3.3 (0.1) ^a
	<i>S. spectabilis</i>	0.3 (0) ^a	5.4 (0.1) ^a	0.2 (0) ^a	6.3 (1.7) ^a	2 (0.3) ^a	2.8 (0.1) ^a
Rhizo-shpere	<i>A. longifolia</i>	0.6 (0.1) ^{bc}	6.2 (0.1) ^b	0.8 (0.1) ^b	32.8 (8.9) ^b	4.7 (1.1) ^a	1.1 (0.1) ^b
	<i>S. spectabilis</i>	1.4 (0.3) ^c	6.2 (0.1) ^b	0.7 (0.1) ^b	37.2 (5.3) ^b	3.3 (0.7) ^a	1.4 (0.4) ^b

rhizospheric and soil fractions underneath the studied plants showed clear differences between the fractions, but no significant differences between species (**table 3.2**). A deviation from this pattern was the OM content, which was higher in the rhizospheric soil fraction of both legumes than in soil of *S. spectabilis*, while soil and rhizospheric soil under *A. longifolia* were not distinguishable. The rhizospheric compartments of both species were slightly less acidic than the surrounding soil, and exhibited 2-fold higher nitrate, 1.7-fold higher soluble P and 6.2-fold higher organic P concentrations, but also 2.4-fold lower ammonia concentrations. Generally, the quantity of rhizospheric soil was highly correlated with the root mass ($r^2 = 0.89$, $p < 0.001$) and was five times higher underneath the invasive species (**table 3.1**). The rhizospheric soil ratio, describing the amount of soil being influenced by roots, was 0.32 in the uppermost 20 cm of soil underneath *A. longifolia*, contrary to 0.07 underneath *S. spectabilis*. Litter mass was not significantly larger underneath the invasive compared to the native species, but litter layers were found to differ significantly in thickness. Total N values did not differ in major tissues, however, *A. longifolia* roots showed a trend for higher total N concentrations compared to *S. spectabilis* roots. Also, while there

were significant differences in the $\delta^{15}\text{N}$ signatures, both species exhibited values close to the atmospheric standard, which is expected in nitrogen fixing legumes. The foliage of the *A. longifolia* was significantly depleted in total P compared to the native *S. spectabilis*, while roots and litter showed only a slightly lower total P in the case of the invasive.

3.4.2 Specific enzyme activities to assess potential flux rates

For rhizosphere, litter and soil, significant differences of specific enzyme activities were found between species (**table 3.3**). The litter produced by the invasive showed 1.5 times higher C related EEA (ΣC : β -glucosidase, β -xylosidase, β -glucuronidase, cellobiohydrolase) than the litter of the native species, however, β -1,4-N-acetylglucosaminidase (Nag) and acid phosphatase (Ap) activities were not significantly different. Differences of specific enzyme activities were more pronounced and significantly different between the rhizospheres of both species, with 2.7-fold higher ΣC , 2.4-fold higher Ap activity and 1.4-fold higher Nag activity underneath the invasive. Root Ap enzyme activities were significantly higher underneath the invasive species, while root β -glucuronidase activities were higher in the native. Ratios of $\ln(\Sigma\text{C}):\ln(\text{Nag})$,

Table 3.3: Specific extracellular enzyme activities of rhizosphere and litter pools, related to organic matter content. ΣC abbreviates the sum of carbon related enzyme activities (β -glucosidase + β -xylosidase + β -glucuronidase + cellobiohydrolase). Also shown are root potential enzyme activities per projected root area. Values are means ($n = 5$) with standard errors in brackets. Rows in bold show significant differences between groups ($p < 0.05$, Wilcoxon test).

		<i>Acacia longifolia</i>	<i>Stauracanthus spectabilis</i>	p - value
Litter specific enzyme activity ($\mu\text{mol h}^{-1} \text{g}^{-1} \text{OM}$)	β-glucosidase	1.9 (0)	1.3 (0)	0.016
	β-xylosidase	0.1 (0)	0 (0)	0.016
	β -glucuronidase	0.1 (0)	0.1 (0)	0.222
	cellobiohydrolase	0.4 (0)	0.3 (0)	0.032
	ΣC	2.5 (0.2)	1.6 (0.1)	0.016
	acid phosphatase (Ap)	1.6 (0)	1.2 (0)	0.151
	N-acetylglucosaminidase (Nag)	1.3 (0)	1 (0)	0.151
Rhizosphere specific enzyme activity ($\mu\text{mol h}^{-1} \text{g}^{-1} \text{OM}$)	β-glucosidase	346.9 (0.3)	103.6 (0.1)	0.016
	β -xylosidase	83.5 (0.1)	40 (0)	0.056
	β -glucuronidase	8 (0)	4.5 (0)	0.095
	cellobiohydrolase	27.8 (0)	14.1 (0)	0.032
	ΣC	466.1 (52.8)	162.2 (21.2)	0.016
	acid phosphatase (Ap)	202.1 (0.2)	93.4 (0.1)	0.008
	N-acetylglucosaminidase (Nag)	16.3 (0)	11.2 (0)	0.008
Root potential enzyme activity ($\text{pmol min}^{-1} \text{mm}^{-2}$)	β -glucosidase	787 (45.3)	752.8 (17.8)	0.222
	($\text{pmol min}^{-1} \text{mm}^{-2}$)	197.7 (5.3)	184 (3.5)	0.095
	β-glucuronidase	10.3 (0.3)	14.3 (1.4)	0.008
	cellobiohydrolase	59.4 (3.3)	55 (1.2)	0.222
	ΣC	1054.4 (52.8)	1006.2 (56.5)	0.151
	acid phosphatase (Ap)	401.2 (3.9)	414.3 (3.7)	0.032
	N-acetylglucosaminidase (Nag)	12.2 (0.6)	14.6 (1.5)	0.31

$\ln(\Sigma C):\ln(\text{Ap})$ and $\ln(\text{Nag}):\ln(\text{Ap})$ can be used for stoichiometry considerations (Sinsabaugh *et al.* 2009), similar to C/N/P ratios in plant tissues. EEA ratios were not significantly different between the species and ranged around 1 in the litter compartment ($\text{SE} \pm 0.1$), while they differed to a larger extent in rhizosphere: $\ln(\Sigma C):\ln(\text{Nag}) = 1.3$, $\ln(\Sigma C):\ln(\text{Ap}) = 1$, $\ln(\text{Nag}):\ln(\text{Ap}) = 0.8$ and the roots: $\ln(\Sigma C):\ln(\text{Nag}) = 2.7$, $\ln(\Sigma C):\ln(\text{Ap}) = 1.1$, $\ln(\text{Nag}):\ln(\text{Ap}) = 0.4$.

3.4.3 Spatial impact

The impact of the legumes on both foliar total N and $\delta^{15}\text{N}$ values of surrounding *C. album* plants was distance dependent and could be modelled using a segmented regression (**figure 3.2**). Both legumes impacted foliar N or $\delta^{15}\text{N}$ of surrounding *Corema album* plants up to certain breakpoint (broken lines) which described the maximum range of influence outside of the canopy. Using this approach, *C. album* plants were grouped into plants growing under the canopy (on the black dotted line, IN), or in distances exceeding the breakpoint, where no significant impact of the legume

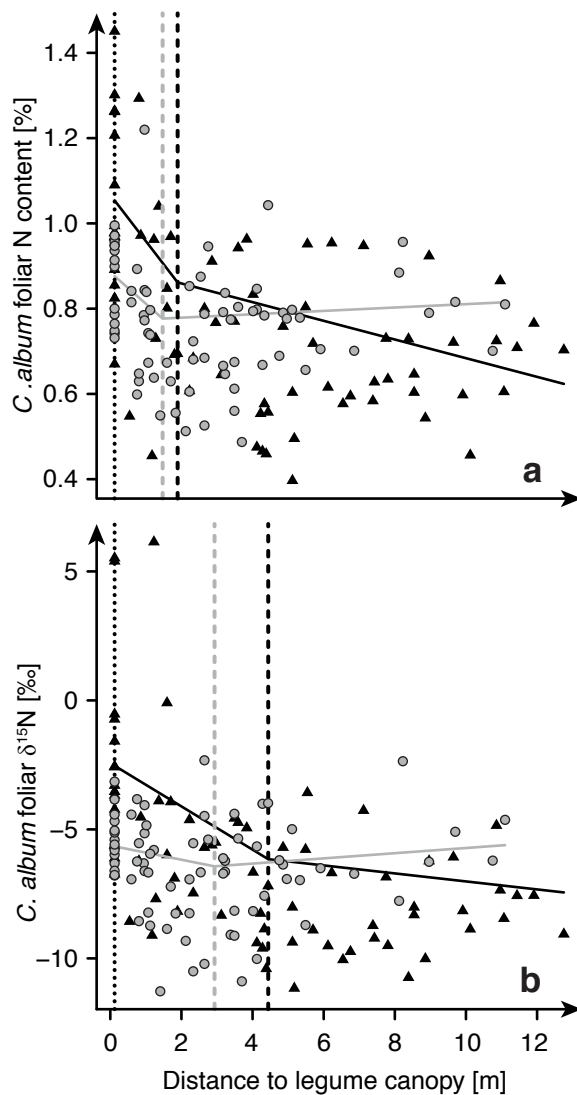


Figure 3.2 Distance effects of invasive and native legumes to *C. album* foliar N and $\delta^{15}\text{N}$. (a) Effect of the distance to the native (*S. spectabilis*) and the invasive (*A. longifolia*) on *C. album* total foliar N. (b) Effect of the distance to the native (*S. spectabilis*) and the invasive (*A. longifolia*) on *C. album* foliar $\delta^{15}\text{N}$. Black triangles indicate *C. album* foliage samples from the surrounding of *A. longifolia*, grey dots samples from the surrounding of *S. spectabilis*. Black dotted lines indicate the canopy of the respective legumes, black and grey broken lines indicate the breakpoint estimated by segmented regression, beyond which the influence of the legumes is not distinguishable from background variation.

was evident (OUT). The influence range of *S. spectabilis* on *C. album* was 1.3 m for foliar total N content and 2.7 m for $\delta^{15}\text{N}$ values, and thus lower than that of *A. longifolia*, with 1.7 m for total N and 4.1 m for $\delta^{15}\text{N}$. Using the radius of the canopy size (table 3.1) and adding the breakpoint distance, the impact range of *A. longifolia* on *C. album* was estimated to

be 1.9-fold greater for foliar N and 3.8-fold greater for foliar $\delta^{15}\text{N}$ than its canopy cover. *S. spectabilis* showed a 3.8-fold larger impact range for impact on *C. album* foliar total N and a 8.2-fold larger impact range on foliar $\delta^{15}\text{N}$. As the impact areas are calculated relative to the canopy, they were larger for the native legume, however, the absolute area of influence was far greater for the invasive plant, 134 m² for *C. album* foliar total N and 250 m² for foliar $\delta^{15}\text{N}$, against 23 m² and 53 m², respectively, for the native.

C. album foliar total N and $\delta^{15}\text{N}$ values were not significantly different between plants growing inside (IN) or outside (OUT) either *A. longifolia* or *S. spectabilis* (figure 3.3) but were generally higher in IN plants compared to OUT plants (Wilcoxon test, $n = 10$, $p < 0.001$). These results were used to express legume influence as % of change in *C. album* total foliar N and ‰ change for *C. album* foliar $\delta^{15}\text{N}$ values (figure 3.3, right boxes). In both cases, *C. album* plants growing in the transects measured around *A. longifolia* show a significantly larger change in total foliar N and foliar $\delta^{15}\text{N}$ values than plants in the transects measured around *S. spectabilis* (Wilcoxon test, $n = 5$, $p < 0.05$).

Contrary to total N and $\delta^{15}\text{N}$ values, no correlation was found between distance to legume canopy and foliar total P concentrations of *C. album*. However, the legume influence on *C. album* foliar total N shows a negative linear relationship ($n = 10$, $r = -0.63$, $p = 0.052$) with *C. album* foliar total P (Fig. 3, left) and legume influence on *C. album* foliar $\delta^{15}\text{N}$ exhibits a significant negative monotonic relationship with foliar total P ($n = 10$, $r_s = -0.673$, $p = 0.033$). Also, *C. album* plants in the transects

measured around *A. longifolia* were significantly lower in foliar P (Wilcoxon test, $n = 5$, $p < 0.05$, **figure 3.4**, right box).

3.4.4 Partial least-squares regression

Using a partial least-squares regression approach (PLSR) with an automatic backward model selection procedure allowed to determine the most important variables predicting the legume influence on the surrounding *C. album* vegetation. Biplots of the first two components showing sample scores and loadings of the variables can be found in **figure 3.3**. The first component of the respective models showed a high correlation with % of change in *C. album* total foliar N ($r^2 = 0.95$), *C. album* total foliar P ($r^2 = 0.67$) and % of change for *C. album* foliar $\delta^{15}\text{N}$ values ($r^2 = 0.91$), while no significant correlation with the second components was evident. Utilising the loadings of the first component, the impact of each variable

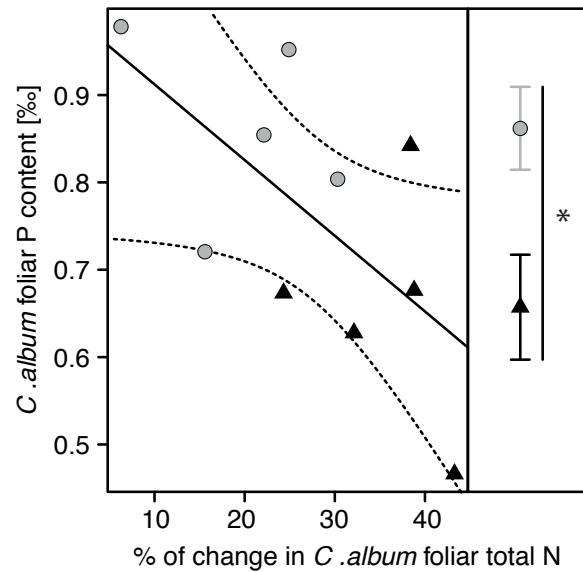


Figure 3.4 Relationship between change in *C. album* foliar total N and P.

On the left: Relationship between % of change in *C. album* foliar total N and foliar total P (‰), dotted lines indicate the 95% confidence interval. Grey dots indicate values for *C. album* plants in the surrounding of *S. spectabilis* and black triangles indicate values from the surrounding of *A. longifolia*. % of change in *C. album* foliar total N was derived from IN and OUT plants as described in **figure 3.3**. *C. album* foliar P content was calculated per legume plant as the mean of all surrounding *C. album* plants ($n = 15$). On the right: *C. album* foliar total P (‰, mean \pm SE, $n = 5$) of all *C. album* plants from five *A. longifolia* (black triangles) and five *S. spectabilis* (grey dots) plants. Asterisks indicate significant differences ($* = p < 0.05$, Wilcoxon test with $n = 5$).

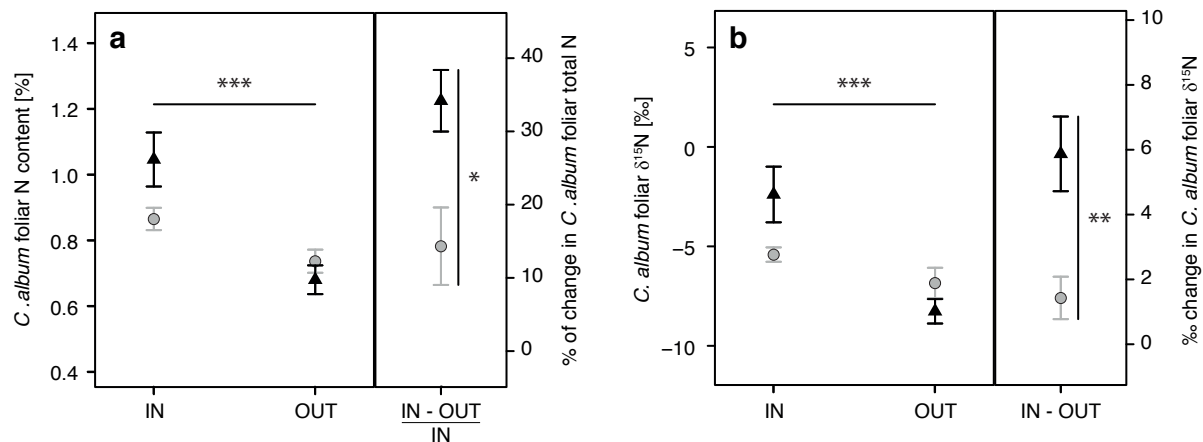


Figure 3.3 Effect of legume presence on *C. album* foliar N and $\delta^{15}\text{N}$.

(a) Effect of legume presence on total foliar N (%) of *C. album* foliage (mean \pm SE, $n = 5$).

(b) Effect of legume presence on $\delta^{15}\text{N}$ (‰) of *C. album* foliage (mean \pm SE, $n = 5$).

Grey dots indicate values for *C. album* plants in the surrounding of *S. spectabilis* and black triangles indicate values from the surrounding of *A. longifolia*. Values for IN and OUT plants were derived from the breakpoint estimation described in **figure 3.2**, with IN values representing plants growing inside the canopy of the respective legume and OUT values being derived from plants growing outside of the legumes influence, i.e. farther away than the breakpoint (left boxes). Legume influence was then described in % of change in *C. album* total foliar N and % change for *C. album* foliar $\delta^{15}\text{N}$ (right boxes). Asterisks indicate significant differences ($* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$; Wilcoxon test with $n = 10$ in left boxes, $n = 5$ in right boxes).

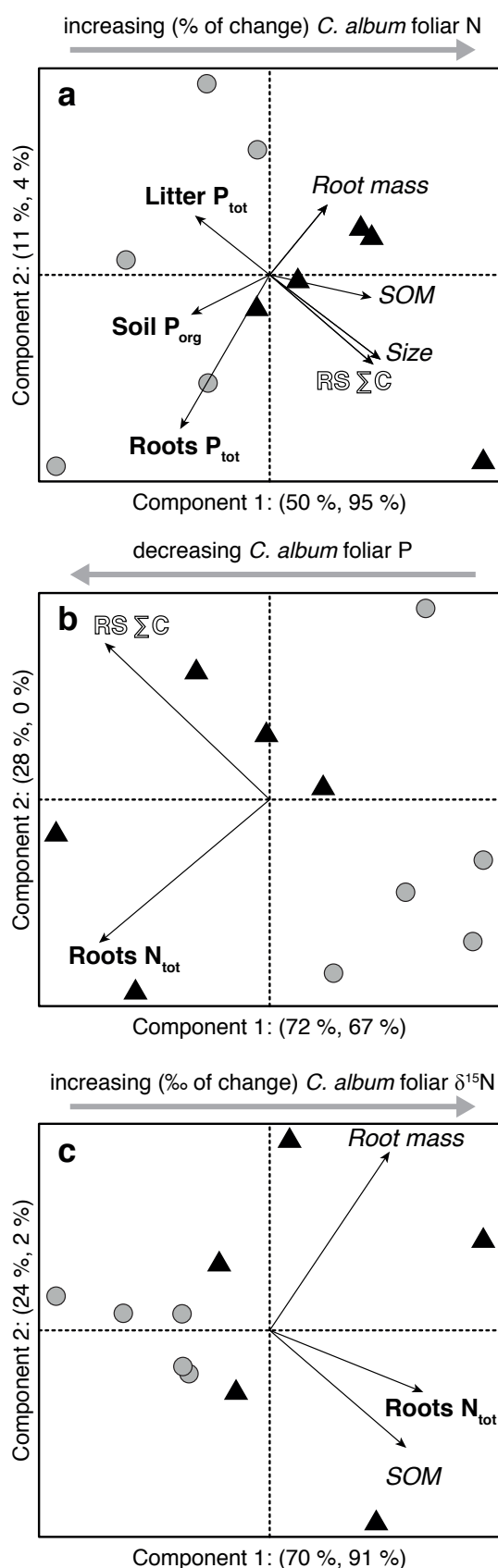


Figure 3.5 Biplots showing sample scores (dots) and loadings of selected variables (labelled arrows) on the first two components of partial least squares regression (PLSR) models. Predicting:

can be expressed as a percentage by calculating the loading weights, where loadings are normalised so that the sum of squares of all loadings within each component is summed to one (Carrascal *et al.* 2009).

In the model describing % of change in *C. album* total foliar N seven variables were retained (**figure 3.5**). Variables related to P contents within the system (total P litter, total P roots and soil organic P) and belowground organic matter pools (SOM and root mass) were the most important predictors, accounting for 41% and 27%, respectively, while C turnover rates in the rhizosphere compartment and plant size each accounted for 16%. The model predicting *C. album* total foliar P (**Fig. 4b**) contained two variables, each accounting for 50% of the variance (root N content and C turnover rates). The model on ‰ of change for *C. album* foliar δ¹⁵N values (**figure 3.5 c**) contained three variables. Here, predictors were soil OM content (33%), root N content (each for 35%), and root mass (31%).

Figure 3.5 (continued)

(a) % of change of *C. album* foliar N

(b) ‰ total *C. album* foliar P

(c) ‰ of change of *C. album* foliar δ¹⁵N

Grey dots indicate samples from the surrounding of *S. spectabilis* and black triangles samples from the surrounding of *A. longifolia*. The models were optimized selecting significant variables by employing an automatic model selection procedure based on variable importance in the projection (VIP) values and significance for prediction. Percentages given in the axis titles are variances in X (predictor space/independent variables) and Y (dependent variable) explained by the respective component. Variables in bold are related to tissue nutrient concentrations, variables in italic are related to biomass and variables with black contours are related to turnover rates. RS = rhizosphere, ΣC = β-glucosidase + β-xylosidase + β-glucuronidase + cellobiohydrolase, P_{tot} = total phosphorus, N_{tot} = total nitrogen, SOM = soil organic matter, P_{org} = organic phosphorus.

3.5 Discussion

Understanding plant invasions requires a thorough analysis of belowground processes, not only in terms of quantitative changes of soil OM pools, but also of nutrient stoichiometry and fluxes (Hulme *et al.* 2013). The work presented here highlights these considerations and adds P as an important nutrient to understand the impact of an ecosystem engineering plant in an oligotrophic model system. Confirming earlier observations (Hellmann *et al.* 2011, Rascher *et al.* 2012) of a spatial *Acacia longifolia* impact on foliar N and $\delta^{15}\text{N}$ of surrounding *Corema album* vegetation (**figure 3.2**), the results furthermore show legume influence on *C. album* N and $\delta^{15}\text{N}$ are negatively correlated with *C. album* foliar P concentrations (**figure 3.4, left**). This fits to the hypothesis of induced N/P imbalance, as it indicates the onset of systemic P depletion while N availability increases. The effect is significantly stronger for the invasive (**figure 3.4, right**), aligning *A. longifolia* with other *Acacia* tree species that have been shown to shift N and P co-limited ecosystems to a stronger P limitation (Sitters *et al.* 2013).

The decrease in *C. album* foliar P was related with higher C turnover rates in the rhizosphere and higher root N (**figure 3.5 b**), while the increase in % of change in *C. album* total foliar N was related with depleted legume tissue P, increased SOM and root mass (**figure 3.5 a**). Both root and rhizosphere mass were increased underneath the invasive *A. longifolia*, compared to the native *Stauracanthus spectabilis* (**table 3.1**). Increased root mass is a known trait of invasive *Acacia* species (Morris *et al.* 2011) and especially important for plant

invasions in low resource environments (Funk 2013). Root N content in turn is involved in mediating microbial C turnover efficiency (Carrillo *et al.* 2014) and microbial nutrient cycling in the rhizosphere (Jones *et al.* 2009). The rhizosphere is crucial for the understanding of plant invasions, due to its role in establishing soil structure (Philippot *et al.* 2013) and as a proxy of plant–plant (Sanon *et al.* 2009) and plant–soil interactions (Callaway *et al.* 2004). The rhizosphere of *A. longifolia* consistently exhibited higher specific EEA (**Table 3**), which indicates higher resource availability and microbial growth (Sinsabaugh *et al.* 2009). In this study, microbial activity, indicated by ΣC EEA values, in the rhizosphere of the legumes was found to be a crucial variable in explaining legume influence on *C. album* N and P (**figure 3.5 a,b**). Rhizosphere β -1,4-N-acetylglucosaminidase (Nag) and Ap activity, were also negatively correlated with legume leaf P (Spearman's rank correlation: $r_s = -0.78$, $p < 0.01$ and $r_s = -0.79$, $p < 0.05$, respectively), which might furthermore indicate a strong P demand of the legumes themselves, especially *A. longifolia*, which additionally exhibited significantly higher values of root Ap.

In contrast to root and rhizosphere mass, litter mass was not different between native and invasive legumes (**table 3.1**), even though *Acacia* plants are known to accumulate thick litter layers underneath their canopy (Marchante *et al.* 2008, Hellmann *et al.* 2011), which is a frequent plant trait associated with ecosystem engineering (Fei *et al.* 2014). Also, litter P was not found to be a significantly related with *C. album* foliar P levels, but had a negative rela-

tion with legume influence on *C. album* foliar N. This could be due to high P-resorbing efficiencies seen in other *Acacia* spp. (He *et al.* 2011) and the potentially poor litter quality of both legumes as indicated by C:N and C:P ratios in the litter (Table 1). Poor substrate quality induces lower C use efficiency of the microbial degrader community (Manzoni *et al.* 2010) and the observed higher Σ C EEA values in the invasive's litter (**table 3.3**) could point to an enhanced litter degradation, which is a frequent occurrence in invaded ecosystems (Liao *et al.* 2008). During litter degradation, N and P are likely released and while in sandy soils around legume canopies soil N has been shown to be influenced in a spatial manner, soluble P did not follow this pattern (Rodríguez *et al.* 2009). The high relevance of increased litter degradation and soil nutrient fluxes during the invasion process is further substantiated by the observation that in its native range *A. longifolia* can itself be out competed by an invasive weed, *Chrysanthemoides monilifera*, which exhibits even higher litter decomposition rates (Lindsay and French, 2005).

Interestingly, while increased Σ C EEA and higher root N values, indicators of increased microbial activity and nutrient turnover, were related with the decrease in *C. album* foliar P, Σ C EEA values increased with legume influence on *C. album* foliar N (**figure 3.5 a**). There is evidence that increased turnover and liberal movement of N between plants could be a mean to decrease competition and increase plant aggregation in a situation where N and P are co-limiting (Teste *et al.* 2015). Indeed, Mediterranean dunes are known to be both very N and P limited (Martínez *et al.* 1998) and extractable P levels of the soil measured here

(**table 3.2**) were half of those reported earlier for similar systems (Marchante *et al.* 2008), thus being indicative of severely nutrient-deficient soil (Funk 2013). However, while liberal movement of N seems to occur in the system observed here (**figure 3.2 a**) which should help sustaining biodiversity (Teste *et al.* 2015), it is known that *A. longifolia* decreases biodiversity during invasion by increasing monospecific plant cover and outcompeting the native vegetation (Marchante *et al.* 2008, Hellmann *et al.* 2011).

This discrepancy might be explained by a high phenotypic plasticity in resource acquisition, coupled with low resource usage in biomass production, which is a strategy of invasive species frequently observed in low-resource ecosystems (Gioria and Osborne 2014). *Acacia longifolia* shows high rhizosphere and litter EEA values and strong investment in belowground biomass, which all contribute to more rapid nutrient cycling and thus resource availability. At the same time, the relation of stronger legume influence on *C. album* N with lower P concentrations in roots and litter (**figure 3.5 a**) could be an indicator for low P usage in biomass production, as *A. longifolia* tissue P concentrations were lower compared to the native legume (**table 3.1**). Species from Australian Mediterranean-type regions often exhibit lower foliar P values as an adaption to their natural low-nutrient habitats (Stock and Verboom 2012). Indeed, *A. longifolia* foliar N concentrations were within range of earlier reports from comparable systems (Marchante *et al.* 2008, Hellmann *et al.* 2011), while foliar P concentrations of the invasive were comparable to values reported for several *Acacia* species under P-impooverished conditions (He *et*

al. 2011). Thus, the resulting foliar N:P ratios between 10 and 20 of the native legume indicate co-limitation for both nutrients (Güsewell 2004) while N:P ratios of *A. longifolia* demonstrate a strong P depletion. However, even though these findings support a P limitation of *A. longifolia*, it is growing to a larger size than the native *S. spectabilis* (**table 3.1**), which is related to increasing legume influence on *C. album* N (**figure 3.5 a**). Differences in canopy size also reflect the different growth forms of the legumes, with *S. spectabilis* growing as a shrub, while *A. longifolia* is creating singular, small trees in this early stage of invasion (Hellmann *et al.* 2011). Furthermore, impact on *C. album* foliar N and $\delta^{15}\text{N}$ is related to high SOM beneath *A. longifolia* (**figure 3.5 a, b**), while in contrast, the native *S. spectabilis* accumulates slowly decaying litter underneath its canopy. Furthermore, being a spiny legume with highly reduced leaves (Barradas *et al.* 1999), *S. spectabilis* likely recycles its shoot N, which would retain N within the plant and make it less available to the surrounding vegetation. This is in line with its foliar $\delta^{15}\text{N}$ values, as N retention can lead to an enrichment in foliar $\delta^{15}\text{N}$ (Unkovich, 2013) and depleted *A. longifolia* foliar $\delta^{15}\text{N}$ values compared to *S. spectabilis* were previously reported (Hellmann *et al.* 2011). Depletion of foliar $\delta^{15}\text{N}$ values could furthermore indicate P deficiency (Lazali *et al.* 2014), however, symbiotic nitrogen fixation is barely affected by insufficient P (Augusto *et al.* 2013), and the work presented here indicates that *A. longifolia*, like other *Acacia* species (Inagaki and Tange 2014), might show a high P use efficiency compared to other N_2 fixing legumes.

In conclusion, the comparison of a native and an invasive legume yielded the most important predictors for their effect on the surrounding vegetation and thus putative reasons for the invasiveness of *A. longifolia* in this system. In contrast to *S. spectabilis*, *A. longifolia* exhibited an efficient P use in biomass production (low P concentrations) and created a below-ground structure consisting of fine roots coupled to a rhizosphere capable of rapid organic matter turnover within its own canopy. In this highly oligotrophic system, the ability of the invasive species to rapidly cycle nutrients may be considered an essential mechanism for its success. As it supplies N to the surrounding vegetation, while requiring substantial amounts of P itself, *A. longifolia* creates an N/P imbalance at community level. Thus, our work gives evidence that the influence on belowground processes and plant–soil-interactions, specifically on community P balance, might substantially contribute to explain the ecosystem engineering character of invasive *Acacia spp.* and their huge ecological impact in dune systems worldwide.

Acknowledgements

Thanks goes to the Estabelecimento Prisional de Pinheiro da Cruz for allowing the establishment of the plots and to Mayele Otte for the help with field work. Also, we would like to thank Vito Muggeo for his quick and helpful replies as well as modifying the function segmented.lme to handle nested random effects. This work was funded by two grants from the European Cooperation in Science and Technology (COST-STSM-ECOST- STSM-ES0806-010512-017110 and COST-STSM-ECOST- STSM-ES0806-011012-023168) as well as grants from the Fundação para a Ciência e Tecnologia (FCT): PTDC/BIA-ECS/ 122214/2010, the PhD grant PD/BD/106061/2015 the project FP7-PEOPLE-2010-IRSES from the Marie Curie International Research Staff Exchange Scheme.

3.6 References

- Augusto, L., Crampon, N., Saur, E., Bakker, M.R., Pellerin, S., Lavaissiere, C.D. and Trichet, P., 2005.** High rates of nitrogen fixation of *Ulex* species in the understory of maritime pine stands and the potential effect of phosphorus fertilization. *Canadian Journal of Forest Research*, 35(5), pp.1183-1192.
- Augusto, L., Delerue, F., Gallet-Budynek, A. and Achat, D.L., 2013.** Global assessment of limitation to symbiotic nitrogen fixation by phosphorus availability in terrestrial ecosystems using a meta-analysis approach. *Global Biogeochemical Cycles*, 27(3), pp.804-815.
- Badano, E.I., Marquet, P.A. and Cavieres, L.A., 2010.** Predicting effects of ecosystem engineering on species richness along primary productivity gradients. *Acta Oecologica*, 36(1), pp.46-54.
- Bellingham, P.J., Walker, L.R. and Wardle, D.A., 2001.** Differential facilitation by a nitrogen-fixing shrub during primary succession influences relative performance of canopy tree species. *Journal of Ecology*, 89(5), pp.861-875.
- Callaway, R.M., Thelen, G.C., Rodriguez, A. and Holben, W.E., 2004.** Soil biota and exotic plant invasion. *Nature*, 427(6976), p.731.
- Carrascal, L.M., Galván, I. and Gordo, O., 2009.** Partial least squares regression as an alternative to current regression methods used in ecology. *Oikos*, 118(5), pp.681-690.
- Carrillo, Y., Dijkstra, F.A., Pendall, E., LeCain, D. and Tucker, C., 2014.** Plant rhizosphere influence on microbial C metabolism: the role of elevated CO₂, N availability and root stoichiometry. *Biogeochemistry*, 117(2-3), pp.229-240.
- Craine, J.M., Elmore, A.J., Aidar, M.P., Bustamante, M., Dawson, T.E., Hobbie, E.A., Kahmen, A., Mack, M.C., McLauchlan, K.K., Michelsen, A. and Nardoto, G.B., 2009.** Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytologist*, 183(4), pp.980-992.
- Cruz, C. and Martins-Loucao, M.A., 2000.** Determination of ammonium concentrations in soils and plant extracts. In: *Nitrogen in a Sustainable Ecosystem*. The Netherlands: Leiden: Backhuys Publishers, pp.291-297.
- D'Angelo, E., Crutchfield, J. and Vandiviere, M., 2001.** Rapid, sensitive, microscale determination of phosphate in water and soil. *Journal of Environmental Quality*, 30(6), pp.2206-2209.
- Díaz Barradas, M.C., Zunzunegui, M., Tirado, R., Ain-Lhout, F. and García Novo, F., 1999.** Plant functional types and ecosystem function in Mediterranean shrubland. *Journal of Vegetation Science*, 10(5), pp.709-716.
- Ehrenfeld, J.G., 2010.** Ecosystem consequences of biological invasions. *Annual Review of Ecology, Evolution, and Systematics*, 41, pp.59-80.
- Fei, S., Phillips, J. and Shouse, M., 2014.** Biogeomorphic impacts of invasive species. *Annual Review of Ecology, Evolution, and Systematics*, 45.
- Funk, J.L., 2013.** The physiology of invasive plants in low-resource environments. *Conservation Physiology*, 1(1).
- Gioria, M. and Osborne, B.A., 2014.** Resource competition in plant invasions: emerging patterns and research needs. *Frontiers in Plant Science*, 5, p.501.
- Güsewell, S., 2004.** N: P ratios in terrestrial plants: variation and functional significance. *New Phytologist*, 164(2), pp.243-266.
- He, H., Bleby, T.M., Veneklaas, E.J. and Lambers, H., 2011.** Dinitrogen-fixing *Acacia* species from phosphorus-impooverished soils resorb leaf phosphorus efficiently. *Plant, Cell & Environment*, 34(12), pp.2060-2070.
- Hellmann, C., Sutter, R., Rascher, K.G., Máguas, C., Correia, O. and Werner, C., 2011.** Impact of an exotic N₂-fixing *Acacia* on composition and N status of a native Mediterranean community. *Acta Oecologica*, 37(1), pp.43-50.
- Hood-Nowotny, R., Umana, N.H.N., Inselbacher, E., Oswald-Lachouani, P. and Wanek, W., 2010.** Alternative methods for measuring inorganic, organic, and total dissolved nitrogen in soil. *Soil Science Society of America Journal*, 74(3), pp.1018-1027.
- Hulme, P.E., Pyšek, P., Jarošík, V., Pergl, J., Schaffner, U. and Vila, M., 2013.** Bias and error in understanding plant invasion impacts. *Trends in Ecology & Evolution*, 28(4), pp.212-218.
- Inagaki, M. and Tange, T., 2014.** Nutrient accumulation in aboveground biomass of planted tropical trees: a meta-analysis. *Soil Science and Plant Nutrition*, 60(4), pp.598-608.
- Jones, D.L., Nguyen, C. and Finlay, R.D., 2009.** Carbon flow in the rhizosphere: carbon trading at the soil-root interface. *Plant and Soil*, 321(1-2), pp.5-33.

- Lazali, M., Bargaz, A., Carlsson, G., Ounane, S.M. and Drevon, J.J., 2014.** Discrimination against ^{15}N among recombinant inbred lines of *Phaseolus vulgaris* L. contrasting in phosphorus use efficiency for nitrogen fixation. *Journal of Plant Physiology*, 171(3-4), pp.199-204.
- Liao, C., Peng, R., Luo, Y., Zhou, X., Wu, X., Fang, C., Chen, J. and Li, B., 2008.** Altered ecosystem carbon and nitrogen cycles by plant invasion: a meta-analysis. *New Phytologist*, 177(3), pp.706-714.
- Lindsay, E.A. and French, K., 2005.** Litterfall and nitrogen cycling following invasion by *Chrysanthemoides monilifera* ssp. *rotundata* in coastal Australia. *Journal of Applied Ecology*, 42(3), pp.556-566.
- Manzoni, S., Trofymow, J.A., Jackson, R.B. and Porporato, A., 2010.** Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. *Ecological Monographs*, 80(1), pp.89-106.
- Marchante, E., Kjoller, A., Struwe, S. and Freitas, H., 2008.** Short-and long-term impacts of *Acacia longifolia* invasion on the belowground processes of a Mediterranean coastal dune ecosystem. *Applied Soil Ecology*, 40(2), pp.210-217.
- Martínez, F., Merino, O., Martín, A., Martín, D.G. and Merino, J., 1998.** Belowground structure and production in a Mediterranean sand dune shrub community. *Plant and Soil*, 201(2), pp.209-216.
- Morris, T.L., Esler, K.J., Barger, N.N., Jacobs, S.M. and Cramer, M.D., 2011.** Ecophysiological traits associated with the competitive ability of invasive Australian acacias. *Diversity and Distributions*, 17(5), pp.898-910.
- Mortimer, P.E., Le Roux, M.R., Pérez-Fernández, M.A., Bénédicto, V.A., Kleinert, A., Xu, J. and Valentine, A.J., 2013.** The dual symbiosis between arbuscular mycorrhiza and nitrogen fixing bacteria benefits the growth and nutrition of the woody invasive legume *Acacia cyclops* under nutrient limiting conditions. *Plant and Soil*, 366(1-2), pp.229-241.
- Muggeo, V.M., Atkins, D.C., Gallop, R.J. and Dimidjian, S., 2014.** Segmented mixed models with random changepoints: a maximum likelihood approach with application to treatment for depression study. *Statistical Modelling*, 14(4), pp.293-313.
- Núñez, M.A. and Dickie, I.A., 2014.** Invasive belowground mutualists of woody plants. *Biological Invasions*, 16(3), pp.645-661.
- Philippot, L., Raaijmakers, J.M., Lemanceau, P. and Van Der Putten, W.H., 2013.** Going back to the roots: the microbial ecology of the rhizosphere. *Nature Reviews Microbiology*, 11(11), p.789.
- Pritsch, K., Courty, P.E., Churin, J.L., Cloutier-Hurteau, B., Ali, M.A., Damon, C., Duchemin, M., Egli, S., Ernst, J., Fraissinet-Tachet, L. and Kuhar, F., 2011.** Optimized assay and storage conditions for enzyme activity profiling of ectomycorrhizae. *Mycorrhiza*, 21(7), pp.589-600.
- R Core Team., 2016.** R: a Language and Environment for Statistical Computing. R. Foundation for Statistical Computing, Vienna, Austria.
- Rascher, K.G., Hellmann, C., Máguas, C. and Werner, C., 2012.** Community scale ^{15}N isoscapes: tracing the spatial impact of an exotic N_2 -fixing invader. *Ecology Letters*, 15(5), pp.484-491.
- Richardson, D.M. and Rejmánek, M., 2011.** Trees and shrubs as invasive alien species—a global review. *Diversity and Distributions*, 17(5), pp.788-809.
- Rodríguez, A., Durán, J., Fernández-Palacios, J.M. and Gallardo, A., 2009.** Spatial pattern and scale of soil N and P fractions under the influence of a leguminous shrub in a *Pinus canariensis* forest. *Geoderma*, 151(3-4), pp.303-310.
- Sanon, A., Andrianjaka, Z.N., Prin, Y., Bally, R., Thioulouse, J., Comte, G. and Duponnois, R., 2009.** Rhizosphere microbiota interferes with plant-plant interactions. *Plant and Soil*, 321(1-2), pp.259-278.
- Sardans, J. and Peñuelas, J., 2012.** The role of plants in the effects of global change on nutrient availability and stoichiometry in the plant-soil system. *Plant physiology*, 160(4), pp.1741-1761.
- Schmidtlein, S., Feilhauer, H. and Bruehlheide, H., 2012.** Mapping plant strategy types using remote sensing. *Journal of Vegetation Science*, 23(3), pp.395-405.
- Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M. and Pyšek, P., 2013.** Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution*, 28(1), pp.58-66.
- Sinsabaugh, R.L., Hill, B.H. and Shah, J.J.F., 2009.** Eoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature*, 462(7274), p.795.
- Sitters, J., Edwards, P.J. and Venterink, H.O., 2013.** Increases of soil C, N, and P pools along an acacia tree density gradient and their effects on trees and grasses. *Ecosystems*, 16(2), pp.347-357.
- Smith, M.S., Fridley, J.D., Goebel, M. and Bauerle, T.L., 2014.** Links between belowground and aboveground resource-related traits reveal species growth strategies that promote invasive advantages. *PloS one*, 9(8), p.e104189.

Stock, W.D. and Verboom, G.A., 2012. Phylogenetic ecology of foliar N and P concentrations and N: P ratios across mediterranean-type ecosystems. *Global Ecology and Biogeography*, 21(12), pp.1147-1156.

Teste, F.P., Veneklaas, E.J., Dixon, K.W. and Lambers, H., 2015. Is nitrogen transfer among plants enhanced by contrasting nutrient-acquisition strategies?. *Plant, Cell & Environment*, 38(1), pp.50-60.

Unkovich, M., 2013. Isotope discrimination provides new insight into biological nitrogen fixation. *New Phytologist*, 198(3), pp.643-646.

Vitousek, P.M., Porder, S., Houlton, B.Z. and Chadwick, O.A., 2010. Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen–phosphorus interactions. *Ecological Applications*, 20(1), pp.5-15.

Watt, M.S., Clinton, P.W., Whitehead, D., Richardson, B., Mason, E.G. and Leckie, A.C., 2003. Above-ground biomass accumulation and nitrogen fixation of broom (*Cytisus scoparius* L.) growing with juvenile *Pinus radiata* on a dryland site. *Forest Ecology and Management*, 184(1-3), pp.93-104.

Chapter 4

Following the degradation of invasive species green waste compost in a sandy soil using novel high throughput microplate methods for enzyme kinetics and pH: Indications for N immobilization, increased soil quality and a crucial role of available P

Ulm, F., Silva, J., Melo, J., Ramos, M., Máguas, C. and Cruz, C., 2019. Following the degradation of invasive species green waste compost in a sandy soil using novel high throughput microplate methods for enzyme kinetics and pH: Indications for N immobilization, increased soil quality and a crucial role of available P. (Accepted in: Soil nitrogen ecology, Springer, Switzerland)

4.1 Abstract

Invasive plant species pose a major threat to many ecosystems worldwide. Especially N-fixing species are problematic, as they can profoundly disrupt soil nutrient cycles, with disastrous effects on soil functioning and native species composition. Eradication measures are often time consuming and expensive, with little incentives for local stakeholders. However, eradication yields large amounts of biomass, which could potentially be composted and used as a soil amendment, incentivizing usage by local farmers and diminishing their spread.

Acacia longifolia is an invasive legume tree, which has recently been successfully composted and used as a growth medium, yet it is not clear how this compost would behave along time if used in an agricultural soil. Also, if it were possible to co-compost foliage and the seed-containing topsoil of *A. longifolia* stands, eradication measures would be more efficient while still producing a viable soil amendment. In the work described here, we assessed the degradation of *A. longifolia* biomass compost (CO) and composted biomass-litter (CL) mix in a sandy soil for ca. 6 months. In order to get a higher resolution of time samples and variables measured, we explored the potential to test a novel high-throughput microplate method that allows for the determination of nutrient concentrations and pH in the soil extract, as well as enzyme kinetics in the same sample.

We found increased apparent V_{\max} values for all enzymes measured in the treatments compared to the control soil, as well as increased pH, decreased inorganic N:P ratios and higher N immobilization. Also, while enzymatic activities diminished along time in the control soil, they increased in the CO treatment, especially in the case of phosphatase, while they were constantly high in the CL treatment. After the addition of 50 mg ammonium per kg soil there was a strong loss of phosphate in the control soil observed, while values in the treatments maintained higher levels, thus keeping the N:P ratios in a range of 10. The final nitrification rates were highly correlated with available P and slightly correlated with N-acetylglucosaminidase K_m , indicating increased microbial activity in the treatments. In conclusion, the method employed here allowed a high resolution of samples and variables to be obtained, which help to explain potential effects of *A. longifolia* GWC in nutrient poor sandy soils. Both GWC types, CO as well as CL, had beneficial effects on the soil quality, as measured by enzymatic activity, increased the pH of this very acidic soil and also immobilized N, potentially decreasing N leaching. However, the CL treatment also had strong nitrification rates, which could counteract the immobilization process. Also, only N was added here and strong interaction effects with available P were found, which on the one hand adds to evidence of high P solubilisation capabilities of the *A. longifolia* litter layer and might be, on the other hand, also a potential way to release locked N in the biomass in an agricultural setting.

4.2 Introduction

Invasive plant species are contributing significantly to human-induced global change due to their strong impacts on biodiversity and ecosystem functioning (Vitousek *et al.*, 1997; Pyšek *et al.*, 2012; Simberloff *et al.*, 2013). While problematic worldwide, Portugal alone is invaded by 8 different invasive *Acacia* spp. (Freitas and Almeida, 2006), with highly detrimental effects on many of its oligotrophic ecosystems. An important species within this group is *Acacia longifolia*, which is also termed an “ecosystem engineer” sensu Badaño *et al.* (2010) and considered “very widespread” globally (Richardson and Rejmánek, 2011). It is one of the most influential invaders in the Portuguese dune systems and adjacent areas, and is completely changing soil functioning during the course of invasion (Marchante *et al.*, 2011). While this problematic is well known, large-scale eradication measures are still lacking behind and are hindered by the fact that many of the *Acacia* spp. exhibit shrub-like growth forms and low timber quality, which makes the economical usage of their biomass difficult. Thus, incentives for the eradication itself should be created, as this would create increasing pressure on the *Acacia* stands, thereby relieving stress on the native vegetation. A promising idea in this field is the production of compost from *Acacia* biomass derived from local eradication measures, which could be used as a soil amendment in adjacent agricultural soils.

The first data available on the composting process of *Acacia* biomass was reported by Brito *et al.* (2013). This work explored the possibility of using *Acacia* compost as an horticultural substrate, replacing more costly and environmentally problematic organic substrates, such as peat (Brito *et al.*, 2015a). Also, comparing this compost as a horticultural substrate with the more common pine bark in an experiment on lettuce growth gave similar results (Brito *et al.*, 2015a), thus making this invasive-derived green waste compost (GWC) an interesting and promising candidate for further research. Furthermore, the usage of slowly degrading biomass, such as pine bark, is also a potential way to increase final compost output (Brito *et al.*, 2015b), which not only increases the mass output of the composting process but is also connected to lower nutrient levels, thus making the final product less suitable as a nutrient source but potentially still useful as an organic amendment for agricultural soils. Also, most of the characteristics of the *Acacia* compost, such as increased C:N ratio and low nutrient contents at a high OM rate, as well as low nutrient and high lignin values could be beneficial for its usage as an organic soil amendment. If these results could be replicated by co-composting *Acacia* biomass with its own litter layer as a slowly degrading biomass, not only would compost output increase, but also the persistent seed bank could be diminished. However, *Acacia* compost has not yet been incorporated into soil and thus it is not known how this amendment would behave, especially in soils that are naturally poor in existing organic matter, such as sandy soils.

The usage of GWC has many potential benefits, such as increased organic matter accumulation and higher nitrate contents (Mugnai *et al.*, 2012), as well as immobilizing mineral N, thereby decreasing N leaching losses and N₂O production by removing mineral N as substrate (Vaughan *et al.*, 2011). The application of GWC can, however, also lead to an increase in nitrification rates, thereby potentially increasing NO₃⁻ concentrations and consequently soil N leaching. This effect was found to be especially strong in acidic soils and is mainly connected to the introduction of ammonium oxidizing bacteria (AOB) into formerly AOB-poor soils (Thangarajan *et al.*, 2015). Thus, apart from changes in the pH levels, the introduction of novel organisms by the soil amendment could especially be problematic when using *Acacia* biomass together with its own topsoil-litter layer for composting. It has been shown that this layer is implicated in N-leakage from the invasive *A. longifolia* to adjacent native vegetation (Ulm *et al.*, 2017), which is mainly connected to organic phosphorus (P) depletion and microbial activity. Higher microbial activity could, on the other hand, also increase mineral N immobilization due to higher microbial growth rates. Heterotrophic microbial growth in the soil, sustained by the degradation of biomass, can only happen if enough P is supplied, as both N and P cycles are tightly coupled (Manzoni *et al.*, 2010). Consequently, not only N and P pools in terms of inorganic nutrient concentrations need to be measured, but also the flux rates of the N, P and carbon (C) cycles, mediated by enzymatic activity, are crucial for the understanding of soil functioning (Sinsabaugh *et al.*, 2009).

Soil enzymatic activities (EA) have long been used as potential indicators of soil quality (Dick, 1994) and subsequent developments made it possible to assess EA at a microscale using fluorometric substrates, which avoids extraction and purification of the product before measurement (Marx *et al.*, 2001). However, there are still several important points to take into account while using these methods in the setting of ecophysiological and soil ecological studies (German *et al.*, 2011). For example, it's imperative to work under substrate saturation conditions in order to assess potential EAs, which is a measure of V_{max} , the maximum reaction rate of an enzyme. If lower than saturating conditions are used, important differences might not be detected (German *et al.*, 2011) and type II error increases in subsequent statistical analysis. Detecting saturation conditions requires, however, producing saturation curves and fitting Michaelis-Menten equations. While this procedure is useful in also providing K_m values, which are a measure of enzyme substrate affinity (Davidson *et al.*, 2006), it is a time consuming procedure, leading many researchers to use a given concentration and not further optimize concentrations (German *et al.*, 2011). Another problem is pH dependence and its measurement, both in the assay itself, as enzymes have specific pH optima (Turner, 2010), and soil pH is often not reported in the publications. Also, it might be the case that pH is different at the microsite where EA was measured and in the bulk soil around the microsite (eg. rhizosphere) (Burns, 1998). Another pH dependent problematic is connected to the measurement of the fluorescent dye (in most cases methylumbelliferone) released by the enzymatic reaction, which fluoresces

best in an alkaline range (DeForest, 2009). As in most cases buffers are used that are not in the range needed for strong fluorescence (pH > 9), NaOH is added to the samples before reading in the fluorometer. However, fluorescence changes along time after the addition of NaOH and the assay buffers used might not behave in the same way upon NaOH addition (DeForest, 2009). Another common drawback of standard methods is the homogenization of soil samples prior to analysis, which can be problematic as several methods lead to cell lysis and the release of intracellular enzymes (German *et al.*, 2011). Lastly, many methods rely on single concentration standards, do not use concentration curves and/or do not take into account the differences in soil types, which, for example, might have different levels of quenching (Wallenstein *et al.*, 2009).

In order to overcome some of the challenges discussed, we used a novel approach to analyse EA in soil samples and tested this methodology on repeated samples of a soil-compost incubation experiment spanning 180 days. The methodology described here was developed from the microplate fluorimetric assay approach for soils as described by Marx *et al.* (2001), and the sequential assay procedure executed in filter microplates for ectomycorrhizal roots as described by Pritsch *et al.* (2011). The usage of filter microplates allows the soil samples to be weighed into the wells with the soil matrix still intact and then performing various types of analysis on the same sample sequentially. Here, we tested washing the soil with distilled water to obtain a soil solution, which subsequently was used for colorimetric pH determination. Also, we created a calibration curve for each well by using increasing

concentrations of methylumbelliferone (MU) and washing out the fluorescent dye before starting the incubation with increasing methylumbelliferyl-substrate concentrations to produce the Michaelis-Menten curves, measuring fluorescence without NaOH addition and higher gains. The large data set created by this methodology was then used to discuss the potential effects of compost addition in sandy agricultural soils.

4.3 Materials and Methods

4.3.1 Soil description, compost production and treatment description

Topsoil (> 15 cm) was sampled in the parish of Longueira/Almograve, Odemira, Portugal (37.68°N, 8.76°W) from an irrigated agricultural land classified as an arenosol (IUSS Working Group, WRB, 2006). Plant material (*Acacia longifolia*) as well as the litter layer below (< 5 cm) were harvested and ground mechanically into pieces < 10 cm and 0.0625 m³ was then composted in custom made insulated aerated cubic containers with a volume of 0.125 m³. The containers were build from ROOFMATE_{TM} extruded polystyrene foam insulation sheets of 5 cm thickness, which were joined using polyurethane foam adhesive spray and sealed with acrylic to maintain temperature and humidity constant.

Plant material was either used pure (Compost) or mixed in a 10:1 (v:v) ratio with the litter layer (CL-mix) and composted for 40 days in controlled mesophilic conditions (40 - 53 °C), keeping volumetric water content above 40 %. Final composted material had: 1.5 N %, 48.0 C % and a C:N ratio of 34.3 for the pure compost and 1.3 N %, 39.7 C % and a C:N ratio of 31.5 for the CL-mix. Composted plant material (Compost and CL-mix), was then incorporated in 500 g soil (n = 5) at a 2 % (w/w) ratio and the mixes incubated at 22° C in the dark in loose-lid aluminium containers. Along the duration of the experiment, soil was kept at field capacity using tap water and samples were taken with a spatula at various time points.

At day 77 of the experiment, 50 mg NH₄⁺ kg⁻¹ soil were added as a solution in distilled water to simulate fertilizer input.

4.3.2 Sampling regime and preparation

Before starting the experiment, subsamples of the soil were oven-dried at 60 °C until constant weight and 500 mg subsequently ashed in a muffle furnace (600°C, 24 h) to determine OM by the loss on ignition method. For soil extracts, subsamples of 5 g were taken and shaken at room temperature (20°C) for 30 min in 20 ml distilled water at days: 1, 21, 73, 77, 97 and 174. After filtering through medical gauze, the extracts were stored at -20°C until usage and, after thawing, extracts were analysed for ammonium, nitrate, nitrite and soluble inorganic phosphorus. For enzyme kinetics and pH measurements, > 1 g subsamples were frozen immediately at -20°C in reaction tubes. Immediately before analysis, samples were thawed at room temperature and weighed into 96-well filter plates (AcroPrep_{TM} 96-filter plate, 350 µl, 30-40 µm PP/PE non-woven media, Pall, Life Sciences, Cramlington, Germany), with ca. 100 mg (± 5 mg) of soil sample per well. For pH analysis, 200 µl ultrapure water (Millipore, Merck, Darmstadt, Germany) was added into each well and shaken (500 rpm) for 30 min. Afterwards, the liquid was transferred to a 250 µl transparent 96 well plate using a vacuum manifold for microplates and frozen at -20°C if not analysed immediately. The wet soil left in the filter plates was subsequently used for the determination of enzyme kinetics.

4.3.3 Colorimetric nutrient analyses of the soil solution

All colorimetric methods were executed in 96-well plates, which were then read in a microplate reader (Rainbow, Tecan, Männedorf, Switzerland). For each single assay day or microplate, a separate calibration curve was prepared in triplicate. The calibration curves were prepared with the respective salts (KH_2PO_4 , $(\text{NH}_4)_2\text{SO}_4$, KNO_3) as serial dilutions in ultrapure water. Ammonium was determined using a modified Berthelot reaction (Cruz and Martins-Loução, 2000), nitrate and nitrite with the VCl_3 /Griess method as described by Hood-Nowotny *et al.* (2010). Soluble inorganic phosphorus was analysed using a malachite-green based microscale method employed as described by D'Angelo *et al.* (2001).

4.3.4 pH analysis

For pH analysis, a novel method was developed, based on a colorimetric microscale method (Newman *et al.* 2012) using Yamada Universal Indicator dye (UI), which was prepared by dissolving 62 mg methyl red, 250 mg bromothymol blue, 25 mg thymol blue and 500 mg phenolphthalein in 500 ml ethanol. The pH was adjusted to 7 with sodium hydroxide and the resulting solution made up to 1 l with H_2O (dest.) and stored in the dark at room temperature. To start the analysis, 20 μl soil solution derived from the filter plates (see above) was mixed with 60 μl H_2O (dest.) and 20 μl UI, mixed thoroughly on the mixer (800 rpm) and absorption then immediately measured at two wavelengths: 545 and 615 nm, which were wavelengths chosen in preexperiments (data not shown). In brief, various pH calibration solutions (pH 4, 5, 6, 7, 8, 9 and 10, produced

with NaOH and HCl in H_2O dest.) were measured at all wavelengths between 400 and 700 nm in 5 nm steps. The resulting absorption curves were then correlated with the pH of the calibration curves using a partial least squares regression approach (see: “statistical analysis” below), which showed a range of wavelengths as potential predictor variables. To simplify the process, only two wavelengths were chosen: 615 nm because it showed the largest amplitude of absorption between the different calibration solutions and 545 because absorption values were the most different to 615 nm. The final linear model for pH prediction was created as: $\text{pH} \sim e^{\text{abs}(615)/\text{abs}(545)}$ and had a correlation coefficient of $r = 0.946$ ($p < 0.001$). To check for interference with higher salt concentrations, calibration solutions were also made in 1 M KCl and measured as described above. Correlation between both was $r = 0.999$ ($p < 0.001$), thus the H_2O calibration curve was used to predict the samples obtained.

4.3.5 Fluorometric analyses

Enzyme kinetics were determined on the wet soil remaining in the filter plates (see above) for the enzymes N-acetylglucosaminidase, β -glucosidase and acid phosphatase using fluorogenic substrates with 4-Methylumbelliferyl (MU) as fluorescent functional group. The substrates MU-N-acetylglucosamine, MU- β -glucoside and MU-phosphate as well as MU were prepared as 10 mM stock solutions in 2-Methoxyethanol and kept at -20°C in the dark until usage. MU calibration solutions (0, 10, 20, 30 and 40 μM) and working solutions for the substrates (0, 62.5, 125, 250, 500, 1000 μM) were prepared in a 0.1 M MES incubation buffer (2-[N-Morpholino]ethane-

sulfonic acid, pH 6.1 with NaOH).

To create a MU calibration curve per soil sample, 200 μ l per well of incubation buffer (IB) was added and fluorescence was measured at 364 nm excitation and 450 nm emission in a fluorescence microplate reader (BIOTEK FLx800, BioTek Instruments, Winooski, USA), setting the gain to 55. Subsequently, vacuum was applied to remove the IB and 200 μ l of the calibration solution with lowest concentration (here 62.5 μ M) was added. After brief shaking on the microplate shaker (500 rpm), fluorescence was measured and the calibration solution removed by applying vacuum. After washing the wells with IB, the next higher concentration was applied and the procedure repeated until the highest concentration point was reached. In order to wash out the MU solution, soil samples were washed with ethanol (80 % in H₂O dest.), as ethanol was reported to quantitatively remove MU from soil and sediment (Boschker and Capenberg, 1994) and because it did not interfere with soil enzyme activity (data not shown). Washing with ethanol was repeated until the baseline values of soil and IB were reached and IB added to start the enzyme kinetic measurements.

Similar to the calibration curve, substrate was added in increasing concentrations, washing with IB between incubation steps. For each step, the samples were incubated for 30 min with the respective substrate concentration and measured at the time points 0, 5, 10, 15, 20, 25 and 30 min. The values obtained for each sample were then used in a linear model to calculate product increase over time (nmole MU min⁻¹) per substrate concentration. This reaction rate was calculated for each concentration

and then correlated with substrate concentrations used with a nonlinear model following the Michaelis Menten equation to estimate apparent substrate affinity (K_M) and apparent maximum rate of activity (V_{max}) for each sample. Before estimation, each saturation curve was checked visually to avoid artefacts.

4.3.6 Statistical analysis

Statistical analysis was performed using R version 3.3.2 (R Core Team, 2016) using package “stats”, if not stated otherwise, and executed on RStudio IDE version 1.0.136. Additional packages used were: “Hmisc” (Harrell *et al.*, 2017), “lawstat” (Hui and Gastwirth, 2008), “lmtree” (Hothorn and Zeileis, 2013), “autopls” (Schmidtlein *et al.*, 2012) and “minpack.lm” (Elzhov *et al.*, 2016). Pairwise comparisons between groups were calculated using pairwise Wilcoxon Rank Sum Tests with Bonferroni - Holm correction. Linear regressions were performed after verifying assumptions using the Breusch-Pagan Test for homoscedasticity and the Shapiro-Wilk Normality Test on the regression model residuals. If assumptions were violated, spearman correlations were employed. The non-linear models (e.g. Michaelis Menten kinetics) were estimated using the *nlsLM()* function from the “minpack.lm” package. Partial least squares with automatic model selection from the “autopls” package was used for predicting the wavelengths best describing pH in the soil solution and for selecting variables best explaining final net nitrification rates.

4.4 Results

4.4.1 Soil solution changes along time

At the beginning of the experiment, nitrate and ammonium concentrations were correlated with soil organic matter (OM) contents ($r_s = -0.7$, $p < 0.01$ and $r_s = 0.55$, $p < 0.05$, respectively), which was lowest in control soil at 0.8 % and similar in both treatments at 2.4 % (Rank Sum Tests with Bonferroni - Holm correction, $p < 0.05$). Before the addition of ammonium, the nitrate concentrations (**figure 4.1, a**) were constantly higher in the soil compared to both treatments. In comparison to the compost (CO), the compost litter treatment (CL) decreased more rapidly (d 21) and both treatments converged before the ammonium addition (d 77) at nearly zero (1.4 and 2.3 mg kg⁻¹, respectively). Conversely, ammonium concentrations (**figure 4.1, b**) were initially higher in both treatments and then all samples converged to low levels (< 0.5 mg kg⁻¹) at d 21. After ammonium addition (d 77, grey vertical

line), nitrate concentrations increased strongly in all samples, with highest values in the soil, intermediate values in the CL treatment and low values in the CO treatment (135, 97 and 51 mg kg⁻¹, respectively). Following an initial increase, the ammonium concentrations declined strongly after the ammonium addition, with the treatments converging to pre-addition levels at d 97 (ca 1.3 mg kg⁻¹) and all samples converging at d 174 (ca 1.3 mg kg⁻¹).

At the start of incubation, both treatments exhibited higher phosphate concentrations than the control soil (**figure 4.2, a**). After addition, on the contrary, there was an initial increase in phosphate in the soil and CL treatment (d 97) and subsequently (d 174) a steep drop in the soil (from 6 to 2.7 mg kg⁻¹). While phosphate values were low in soil and intermediate in CO treatment at the end of the experiment, CL values were the highest (8.3 mg kg⁻¹) and phosphate concentrations increased in both

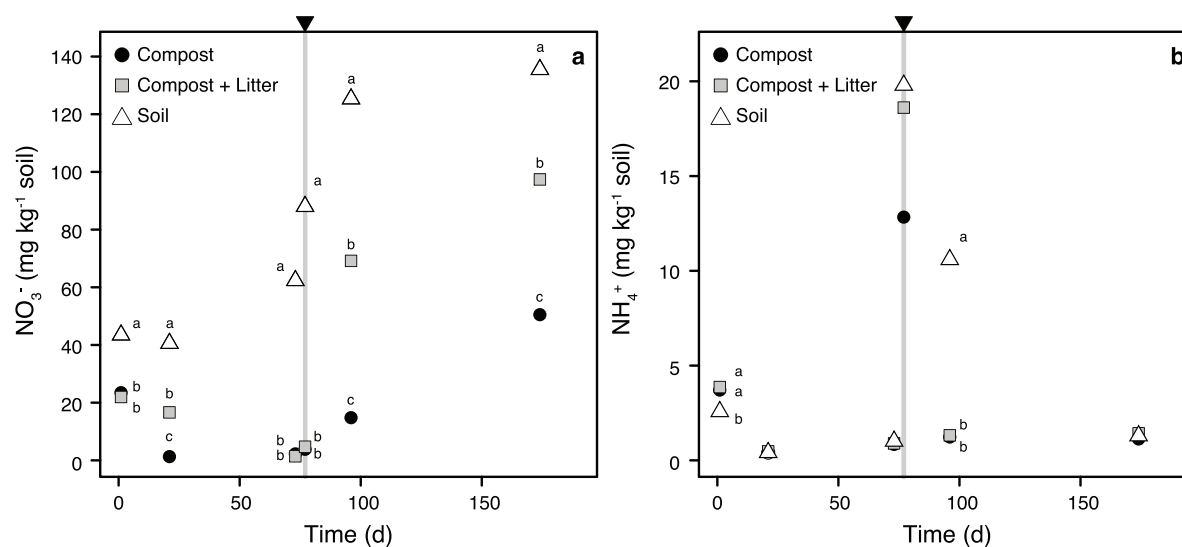


Figure 4.1 Changes in nitrate and ammonium concentrations along time

(a) Changes in nitrate concentrations in soil extracts along time.

(b) Changes in ammonium concentrations in soil extracts along time.

Letters indicate differences between treatments (pairwise Wilcoxon test, $n = 5$, $p < 0.05$). The grey line indicates the addition of 50 mg ammonium per kg soil.

CO and CL constantly during the experiment ($r_s = 0.51$, $p < 0.01$, $n = 30$; $r_s = 0.74$, $p < 0.001$, $n = 30$). The soluble N:P ratios (figure 4.2, b), calculated using phosphate and the sum of nitrate and ammonium, was, contrary to the fluctuations in phosphate concentrations, constantly higher in the soil samples compared to the treatments. The greatest difference was found at the end of the experiment (d 174), with an N:P ratio of 58 in the soil, contrary to 6 and 9 for CO and CL treatments, respectively. The pH values were in general in the acidic range, always below 4.4 (figure 4.3, a) and were decreasing over time, with strongest decreases found for the soil, followed by the CO and CL treatments ($r_s = -0.8$, -0.57 , -0.58 , respectively, all $p < 0.001$, $n = 30$).

4.4.2 Changes in V_{max} and K_m

For all enzymes measured, apparent V_{max} (figure 4.3 b, c, d) and apparent K_m were highly correlated ($r_s > 0.9$, $p < 0.001$, $n = 90$), thus, figure 4.3 shows only apparent V_{max} values. In general, soil shows significantly lower apparent V_{max} values along time, while CO and CL treatments showed a similar pattern. Along time, apparent V_{max} decreased in soil, with strongest decreases measured for β -glucosidase ($r_s = -0.84$, $p < 0.001$, $n = 30$) and acid phosphatase ($r_s = -0.57$, $p < 0.01$, $n = 30$) and smaller decreases for N-acetylglucosaminidase ($r_s = -0.45$, $p < 0.05$, $n = 30$). On the contrary, apparent V_{max} did not significantly change over time in the CL treatment, while both acid phosphatase and N-acetylglucosaminidase ($r_s = 0.44$ and $r_s = 0.38$, respectively, $p < 0.05$, $n = 30$) apparent V_{max} were increasing in the CO treatment. The patterns observed for apparent K_m values were comparable to the apparent V_{max} values, with both β -glucosidase and N-acetylglucosaminidase

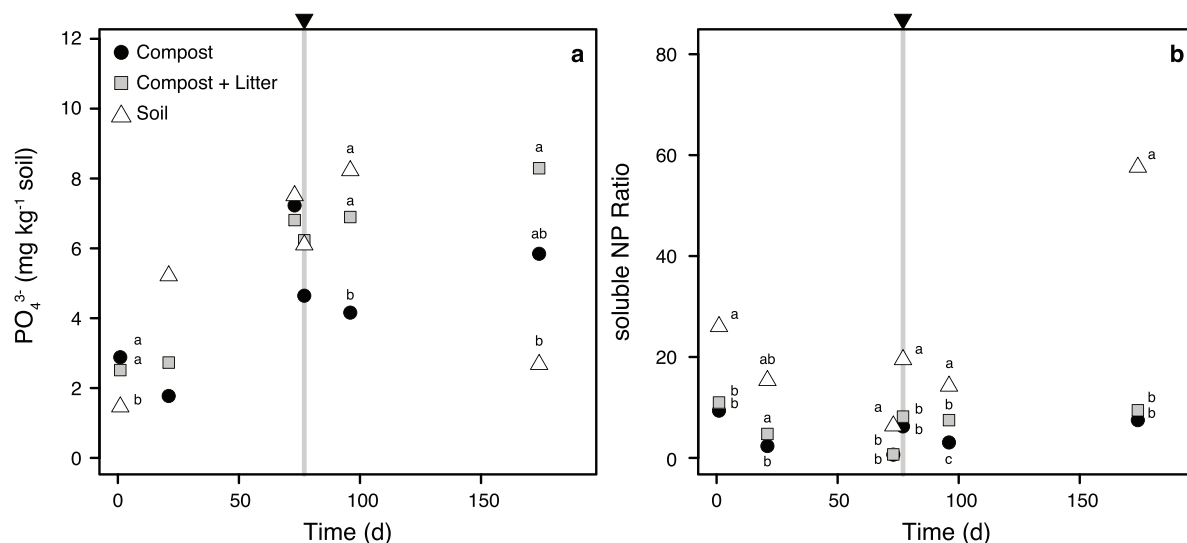


Figure 4.2 Changes in phosphate concentrations and N:P ratios in the soil extract along time.

(a) Changes in phosphate concentrations in soil extracts along time.

(b) Changes in N:P ratios in soil extracts along time.

Letters indicate differences between treatments (pairwise Wilcoxon test, $n = 5$, $p < 0.05$). The grey line indicates the addition of 50 mg ammonium per kg soil.

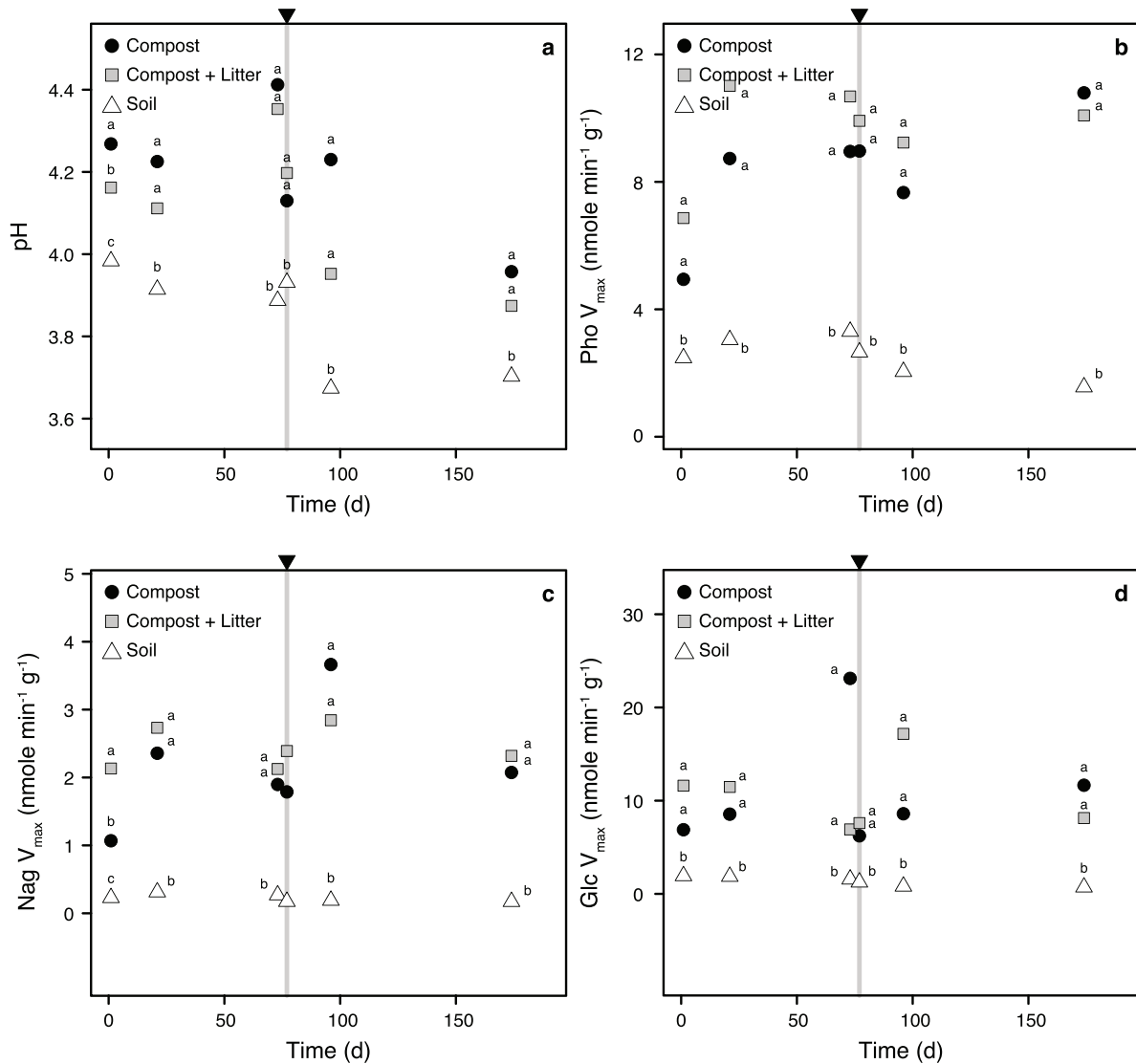


Figure 4.3 Changes in pH and maximum rate of activity (V_{max}) of various enzymes. (a) Changes in pH in soil extracts along time. (b) Changes in maximum rate of activity (V_{max}) for Pho = phosphatase along time. (c) Changes in maximum rate of activity (V_{max}) for Nag = N-acetylglucosaminidase along time. (d) Changes in maximum rate of activity (V_{max}) for Glc = β -glucosidase along time. Letters indicate differences between treatments (pairwise Wilcoxon test, $n = 5$, $p < 0.05$). The grey line indicates the addition of 50 mg ammonium per kg soil.

decreasing along time in the control soil ($r_s = -0.45$ and $r_s = -0.43$, respectively, $p < 0.05$, $n = 30$). Similar to the apparent V_{max} values, acid phosphatase and N-acetylglucosaminidase ($r_s = 0.45$ and $r_s = 0.43$, respectively, $p < 0.05$, $n = 30$) increased in the CO treatments and, contrary to the apparent V_{max} values, N-acetylglucosaminidase apparent K_m values also increased in the CL treatment ($r_s = 0.48$, $p < 0.01$, $n = 30$).

4.4.3 Net nitrification rates and correlations

After addition of 50 mg NH_4^+ per kg soil (d 77), the percentage of N nitrified to NO_3^- was calculated by setting the NO_3^- to 0 and calculating the percentage of change until the end of the experiment (d 174) (figure 4.4). At day 96, samples from the CO exhibited the lowest net nitrification rate at 7 %, with intermediate values in the soil (22 %) and the highest

rates in the CL treatment (37 %). At day 174, CO and soil values converge at 27 %, while net nitrification rates in the CL treatment are significantly higher at 54 %. By employing a PLSR on the last nitrification values (d 174) as a proxy for final nitrification rates, of 9 variables employed for selection (PO_4^{3-} , N:P ratio, pH, and apparent V_{\max} and K_m values of all enzymes measured), PO_4^{3-} and apparent K_m values of N-acetylglucosaminidase were selected as the main variables explaining the final net nitrification rates ($r^2 = 0.44$). Both variables were also correlated with the % of NH_4^+ - N nitrified, with a stronger correlation found between NH_4^+ - N nitrified and PO_4^{3-} (figure 4.5, a) than for apparent K_m values of N-acetylglucosaminidase (figure 4.5, b).

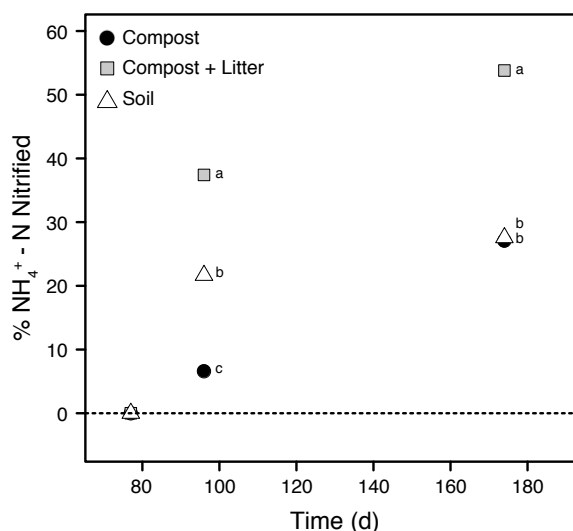


Figure 4.4 Amount of NH_4^+ - N nitrified to NO_3^- after adding 50 mg NH_4^+ per kg soil. Letters indicate differences between treatments (pairwise Wilcoxon test, $n = 5$, $p < 0.05$).

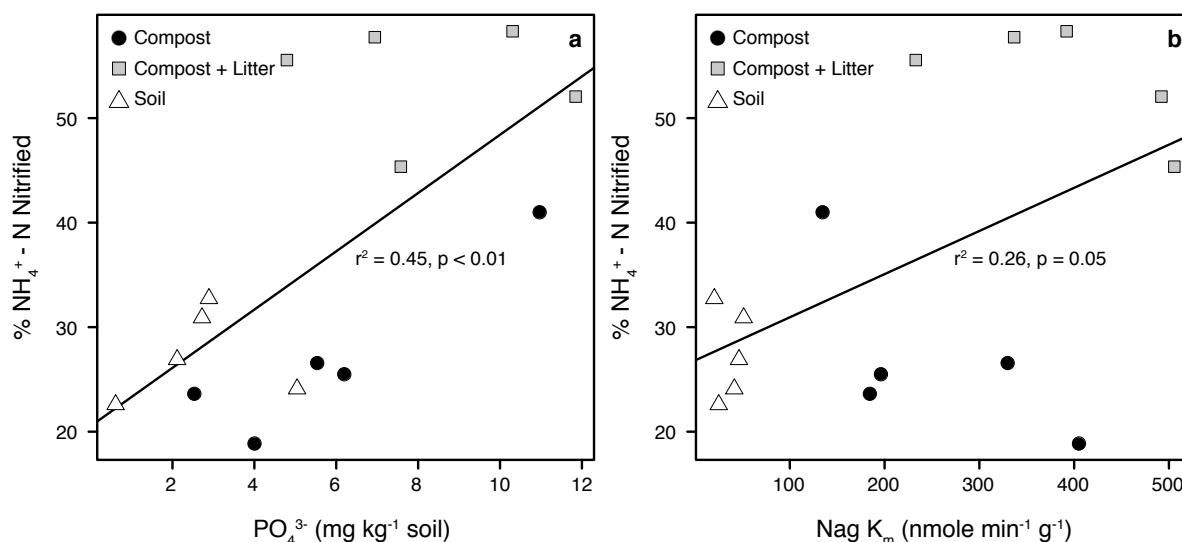


Figure 4.5 Correlations of NH_4^+ - N nitrified to NO_3^- at day 174 with PO_4^{3-} concentration and apparent K_m of Nag = N-acetylglucosaminidase

(a) Pearson correlation of NH_4^+ - N nitrified to NO_3^- at day 174 and PO_4^{3-} concentration.

(b) Pearson correlation of NH_4^+ - N nitrified to NO_3^- at day 174 and apparent K_m of Nag = N-acetylglucosaminidase.

4.5 Discussion

As expected, green waste compost (GWC) application increased soil organic matter concentrations, which is accordance with literature (e.g. Mugnai *et al.*, 2012). GWC additions also led to decreased mineral N (both NO_3^- and NH_4^+ , **figure 4.1 a, b**), probably by immobilizing N in the microbial biomass (Vaughan *et al.*, 2011). After addition of 50 mg NH_4^+ per kg soil, there was a rapid increase in NO_3^- detectable, with the largest absolute concentrations measured in the control soil. While net nitrification was high, the setup of the experiment did not allow to conclusively determine gross nitrification rates, as gross nitrate consumption was not measured and potential nitrate immobilization by the microbial community can be very high in certain soils (Stark and Hart, 1997). In terms of ammonium concentrations, both treatment soils showed a rapid decline and reached minimum concentrations already after 19 days, whereas the control soil reached minimum concentrations only after 97 days (Fig. 1 b). This could be due to low activity of ammonium oxidizing bacteria in the control soil, which might have been introduced in both treatment soils by the GWC addition (Thangarajan *et al.*, 2015). Interestingly, while the absolute concentrations of NO_3^- were the highest in the control soil at the end of the experiment, the largest increases in NH_4^+ nitrified after the addition of 50 mg NH_4^+ per kg soil were found in the compost-litter (CL) treatment, with similar rates for both control soil and compost amended soil (CO) (**figure 4.4**). This could be a further indication for the presence of soil microorganisms from the invasives' species topsoil, which survived

the mesothermic composting process, as it is known that *A. longifolia* topsoil exhibits far higher potential nitrification rates than non-invaded soil (Marchante *et al.*, 2008). On the contrary, there was a slowing effect on nitrification in the CO treatment, which could be connected to the introduction of carbon (C) rich material lacking sufficient available N for its own degradation, thus immobilizing N and making it unavailable for nitrification (Vaughan *et al.*, 2011). Indeed, both amendments used here exhibit high C:N ratios, which make them materials with high potential to immobilize N (Chaves *et al.*, 2005).

Apart from the immobilization itself, there might be other factors explaining differences in nitrification and mineral N concentrations in general. Of great importance for nitrification is for example soil pH, as the substrate for the ammonia monooxygenase enzyme, which is responsible for the initial step in ammonia oxidation, is NH_3 and not NH_4^+ , posing serious problems for nitrification in acid soil environments (Norton and Stark, 2011). Using a novel colorimetric method for pH determination, we were able to follow pH changes in the samples also used for enzymatic activity (EA) measurements (**figure 4.3 a**). While pH values were acidic in general (< 4.5), they were especially low in the control soil, which could explain lower nitrification rates. Particularly after ammonium addition, pH values further dropped, however, nitrification has been reported to occur in soils as acidic as pH 3 (De Boer and Kowalchuk, 2001), which was not reached in the course of this experiment.

Contrary to the pH values, which showed a similar trend in the control soil as well as in the treatments, EA assays showed opposite trends between control soil and treatments (**figure 4.3 b, c, d**). In general, K_m and V_{max} values were highly correlated, which is common in ecological studies of enzymology, as enzymatic substrate turnover in complex microbial populations does not follow Michaelis-Menten kinetics (Williams, 1973). This is mainly attributed to control mechanisms linked to substrate availability and microbial interactions, operating from molecular to population level (Lugtenberg *et al.*, 2002) and thus values obtained here are termed “apparent K_m ” and “apparent V_{max} ”. Generally, apparent K_m is considered a “relative measure of substrate concentration” and apparent V_{max} a “relative measure of enzyme abundance” (Wallenstein *et al.*, 2010). In the experimental setup used here, no further substrates were added after setting up the incubation, therefore any changes in apparent K_m or V_{max} could only be attributed to the degradation of existing biomass. Indeed, both treatments showed consistently higher apparent V_{max} , indicating higher enzyme abundance and thus microbial activity. This further substantiates the hypothesis of N immobilization by microbial growth while also indicating improved soil quality (Dick, 1994). Especially apparent V_{max} of β -glucosidase, which is already lower in the control soil at the beginning of the experiment, decreases further along the experiment, a change that could be explained by the strong pH dependence of this enzyme (Acosta-Martinez and Tabatabai, 2000). However, if that were the case, acid phosphatase should increase in activity (Acosta-Martinez and Tabatabai, 2000) and the opposite pattern

was observed (**figure 4.3 b**). Also N-acetylglucosaminidase activity, a marker for fungal activity in the soil (Miller *et al.*, 1998), declines (**figure 4.3 c**), thus indicating that the decreases in apparent V_{max} are probably related with diminished enzyme abundance due to the reduction in overall degradation activity. In contrast, apparent V_{max} of acid phosphatase and N-acetylglucosaminidase do increase along time in the CO treatment and while apparent V_{max} does not change in the CL treatment along time, it is not statistically different from the CO treatment at any time. The increase in V_{max} and in K_m of N-acetylglucosaminidase should therefore be mainly due to higher microbial activity derived from microbial degradation of the introduced biomass. This is in accordance with the usage of N-acetylglucosaminidase activity as a proxy for N mineralization (Ekenler and Tabatabai, 2002) and, as the degradation of C rich material requires both N and P to proceed (Manzoni *et al.*, 2010), connects with the higher acid phosphatase activities observed.

Indeed, the phosphate concentrations were similar between all treatments until the addition of ammonium (**figure 4.2 a**) but then they decreased markedly in the CO treatment and the control soil compared to the CL treatment. This could suggest a lack in available phosphate, which is an indication further substantiated by the strong increase in soluble N:P ratios in the control soil (**figure 4.2 b**). While it is difficult to conclude N or P limitation just from soluble soil nutrient status, it is known that N:P ratios above 30 suggest strong P limitation in algae (Rhee, 1978), a value far exceeded at the final time point in the control soil. That P limitation might be an important

factor for organic matter degradation in this experiment is also substantiated by the coupling of phosphate concentration to the final nitrification rates (**figure 4.5 a**). This finding is in line with potential P limitation by the nitrifying microbial community in P poor soils (He and Dijkstra, 2015), as microbial growth requires large amounts of P for RNA production during degradation activity (Franklin *et al.*, 2011). Apart from the correlation with phosphate, only K_m of N-acetylglucosaminidase was correlated with the final nitrification rates. N-acetylglucosaminidase is an enzyme highly correlated with fungal activity (Miller *et al.*, 1998), which are known to be able to perform heterotrophic nitrification, especially under acidic soil conditions (De Boer and Kowalchuk, 2001). As fungi are also prolific P scavengers with efficient phosphatases for a variety of organic P compounds (Tarafdar *et al.*, 2001), it might be assumed that fungi are the most important players in the nitrification pattern observed, at least in the latter stage of the experiment. Thus, they might also be the main organisms involved in the prior process of N immobilization and should be further investigated in terms of community structure and functional traits to understand the mechanisms at work and how to employ them for a selective nutrient supply to crop plants.

In conclusion, the methods for pH and enzyme kinetics profiling showed good preliminary results. Apart from leaving the soil sample intact, this novel method for enzymatic analysis allowed for a high throughput analysis of small soil samples and could be combined with prior nutrient extraction directly from the filter plates. The colorimetric pH analysis in

turn, apart from also being useful in combination with the enzymatic method, can also help to screen soils from very heterogeneous environments and could precede a more established method, such as the more time consuming measurement with an electrode. While both methodologies need to be further tested in various other soils before large scale implementation, they could allow to analyse soil microsites in a time effective manner, thus increasing resolution in soil enzymatic tests.

Using these methods, together with other high throughput colorimetric assays for nutrients in soil extraction, we were able to assess some benefits and potential pitfalls of *A. longifolia* GWC application in nutrient poor sandy soils. While soil quality, as measured by enzymatic activity, clearly increased upon treatment and also increased pH and P availability was observed, there are still open questions regarding the microbial mechanisms, especially observed in the CL treatment. However, the GWC application as soil amendment could potentially decrease nutrient leaching, decrease the demand in mineral fertilizer and increase soil health. In order to achieve this, more studies will be needed to predictably immobilize and remobilize N and P in an agricultural setting, benefitting soil microorganisms, the crop and the surrounding ecosystems at the same time.

Acknowledgements

We want to thank Sousa Prado & Filhos - Agro-Pecuária, Lda for the logistical and technical support in the field. This work was funded by the PhD grant PD/BD/106061/2015.

4.6 References

- Acosta-Martinez, V. and Tabatabai, M.A., 2000.** Enzyme activities in a limed agricultural soil. *Biology and Fertility of Soils*, 31(1), pp.85-91.
- de Almeida, J.D. and Freitas, H., 2006.** Exotic naturalized flora of continental Portugal-A reassessment. *Botanica Complutensis*, 30, p.117.
- Badano, E.I., Marquet, P.A. and Cavieres, L.A., 2010.** Predicting effects of ecosystem engineering on species richness along primary productivity gradients. *Acta Oecologica*, 36(1), pp.46-54.
- Boschker, H.T.S. and Cappenberg, T.E., 1994.** A sensitive method using 4-methylumbelliferyl- β -cellobiose as a substrate to measure (1, 4)- β -glucanase activity in sediments. *Applied and Environmental Microbiology*, 60(10), pp.3592-3596.
- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S., 2013.** Composting for management and resource recovery of invasive *Acacia* species. *Waste Management & Research*, 31(11), pp.1125-1132.
- Brito, L.M., Reis, M., Mourão, I. and Coutinho, J., 2015. (a)** Use of acacia waste compost as an alternative component for horticultural substrates. *Communications in Soil Science and Plant Analysis*, 46(14), pp.1814-1826.
- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S.R., 2015. (b)** Co-composting of invasive *Acacia longifolia* with pine bark for horticultural use. *Environmental Technology*, 36(13), pp.1632-1642.
- Burns, R.G., 1982.** Enzyme activity in soil: location and a possible role in microbial ecology. *Soil Biology and Biochemistry*, 14(5), pp.423-427.
- Chaves, B., De Neve, S., Boeckx, P., Van Cleemput, O. and Hofman, G., 2005.** Screening organic biological wastes for their potential to manipulate the N release from N-rich vegetable crop residues in soil. *Agriculture, Ecosystems & Environment*, 111(1-4), pp.81-92.
- Cruz, C. and Martins-Loucao, M.A., 2000.** Determination of ammonium concentrations in soils and plant extracts. In: *Nitrogen in a Sustainable Ecosystem*. The Netherlands: Leiden: Backhuys Publishers, pp.291-297.
- Davidson, E.A., Janssens, I.A. and Luo, Y., 2006.** On the variability of respiration in terrestrial ecosystems: moving beyond Q_{10} . *Global Change Biology*, 12(2), pp.154-164.
- D'Angelo, E., Crutchfield, J. and Vandiviere, M., 2001.** Rapid, sensitive, microscale determination of phosphate in water and soil. *Journal of Environmental Quality*, 30(6), pp.2206-2209.
- De Boer, W. and Kowalchuk, G.A., 2001.** Nitrification in acid soils: microorganisms and mechanisms. *Soil Biology and Biochemistry*, 33(7-8), pp.853-866.
- DeForest, J.L., 2009.** The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and L-DOPA. *Soil Biology and Biochemistry*, 41(6), pp.1180-1186.
- Dick R.P., 1994.** Soil enzyme activity as indicators of soil quality. In: *Defining Soil Quality for a Sustainable Environment*, SSSA Special Publication 35. Soil Science Society of America, Madison, Wisconsin, pp. 107-124.
- Ekenler, M. and Tabatabai, M., 2002.** β -Glucosaminidase activity of soils: effect of cropping systems and its relationship to nitrogen mineralization. *Biology and Fertility of Soils*, 36(5), pp.367-376.
- Elzhov, T.V., Mullen, K.M., Spiess, A. and Bolker, B., 2016.** R interface to the Levenberg-Marquardt non-linear least-squares algorithm found in MINPACK. Plus Support for Bounds. <https://CRAN.R-project.org/package=minpack.lm>
- Franklin, O., Hall, E.K., Kaiser, C., Battin, T.J. and Richter, A., 2011.** Optimization of biomass composition explains microbial growth-stoichiometry relationships. *The American Naturalist*, 177(2), pp.29-42.
- German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L. and Allison, S.D., 2011.** Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry*, 43(7), pp.1387-1397.
- Harrell, F.E., with contributions from Charles Dupont and many others., 2017.** "Hmisc: Harrell Miscellaneous". R package version 4.0-1.
- He, M. and Dijkstra, F.A., 2015.** Phosphorus addition enhances loss of nitrogen in a phosphorus-poor soil. *Soil Biology and Biochemistry*, 82, pp.99-106.
- Hood-Nowotny, R., Umana, N.H.N., Inselbacher, E., Oswald-Lachouani, P. and Wanek, W., 2010.** Alternative methods for measuring inorganic, organic, and total dissolved nitrogen in soil. *Soil Science Society of America Journal*, 74(3), pp.1018-1027.
- Hothorn, T., Zeileis, A., 2013.** Lmtest: Diagnostic Checking in Regression Relationships. <http://CRAN.R-project.org/package=lmtest>
- Hui, W., Gel, Y.R. and Gastwirth, J.L., 2008.** lawstat: an R package for law, public policy and biostatistics. *Journal of Statistical Software*, 28(3), pp.1-26. <http://CRAN.R-project.org/package=lawstat>

- IUSS Working Group, WRB., 2006.** World reference base for soil resources. World Soil Resources Report, 103.
- Lugtenberg, B.J., Chin-A-Woeng, T.F. and Bloembergen, G.V., 2002.** Microbe–plant interactions: principles and mechanisms. *Antonie Van Leeuwenhoek*, 81(1-4), pp.373-383.
- Manzoni, S., Trofymow, J.A., Jackson, R.B. and Porporato, A., 2010.** Stoichiometric controls on carbon, nitrogen, and phosphorus dynamics in decomposing litter. *Ecological Monographs*, 80(1), pp.89-106.
- Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2008.** Short-and long-term impacts of *Acacia longifolia* invasion on the belowground processes of a Mediterranean coastal dune ecosystem. *Applied Soil Ecology*, 40(2), pp.210-217.
- Marchante, H., Freitas, H. and Hoffmann, J.H., 2011.** Post-clearing recovery of coastal dunes invaded by *Acacia longifolia*: is duration of invasion relevant for management success? *Journal of Applied Ecology*, 48(5), pp.1295-1304.
- Marx, M.C., Wood, M. and Jarvis, S.C., 2001.** A microplate fluorimetric assay for the study of enzyme diversity in soils. *Soil Biology and Biochemistry*, 33(12-13), pp.1633-1640.
- Miller, M., Palojarvi, A., Rangger, A., Reeslev, M. and Kjeller, A., 1998.** The use of fluorogenic substrates to measure fungal presence and activity in soil. *Applied and Environmental Microbiology*, 64(2), pp.613-617.
- Mugnai, S., Masi, E., Azzarello, E. and Mancuso, S., 2012.** Influence of long-term application of green waste compost on soil characteristics and growth, yield and quality of grape (*Vitis vinifera* L.). *Compost Science & Utilization*, 20(1), pp.29-33.
- Newman, J., Sayle, R.A. and Fazio, V.J., 2012.** A universal indicator dye pH assay for crystallization solutions and other high-throughput applications. *Acta Crystallographica Section D: Biological Crystallography*, 68(8), pp.1003-1009.
- Norton, J.M. and Stark, J.M., 2011.** Regulation and measurement of nitrification in terrestrial systems. *Methods in Enzymology* (486), pp.343-368.
- Pritsch, K., Courty, P.E., Churin, J.L., Cloutier-Hurteau, B., Ali, M.A., Damon, C., Duchemin, M., Egli, S., Ernst, J., Fraissinet-Tachet, L. and Kuhar, F., 2011.** Optimized assay and storage conditions for enzyme activity profiling of ectomycorrhizae. *Mycorrhiza*, 21(7), pp.589-600.
- Pyšek, P., Jarošík, V., Hulme, P.E., Pergl, J., Hejda, M., Schaffner, U. and Vilà, M., 2012.** A global assessment of invasive plant impacts on resident species, communities and ecosystems: the interaction of impact measures, invading species' traits and environment. *Global Change Biology*, 18(5), pp.1725-1737.
- R Core Team., 2016.** R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>
- Rhee, G.Y., 1978.** Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake 1. *Limnology and Oceanography*, 23(1), pp.10-25.
- Richardson, D.M. and Rejmánek, M., 2011.** Trees and shrubs as invasive alien species—a global review. *Diversity and Distributions*, 17(5), pp.788-809.
- Schmidtlein, S., Feilhauer, H. and Bruehlheide H., 2012.** Mapping plant strategy types using remote sensing. *Journal of Vegetation Science* (26) pp.395-405.
- Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M. and Pyšek, P., 2013.** Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution*, 28(1), pp.58-66.
- Sinsabaugh, R.L., Hill, B.H. and Shah, J.J.F., 2009.** Eoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment. *Nature*, 462(7274), p.795.
- Stark, J.M. and Hart, S.C., 1997.** High rates of nitrification and nitrate turnover in undisturbed coniferous forests. *Nature*, 385(6611), p.61.
- Tarafdar, J.C., Yadav, R.S. and Meena, S.C., 2001.** Comparative efficiency of acid phosphatase originated from plant and fungal sources. *Journal of Plant Nutrition and Soil Science*, 164(3), pp.279-282.
- Thangarajan, R., Bolan, N.S., Naidu, R. and Surapaneni, A., 2015.** Effects of temperature and amendments on nitrogen mineralization in selected Australian soils. *Environmental Science and Pollution Research*, 22(12), pp.8843-8854.
- Turner, B.L., 2010.** Variation in pH optima of hydrolytic enzyme activities in tropical rain forest soils. *Applied and Environmental Microbiology*, 76(19), pp.6485-6493.
- Ulm, F., Hellmann, C., Cruz, C. and Máguas, C., 2017.** N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume. *Oikos*, 126(2).

Vaughan, S.M., Dalal, R.C., Harper, S.M. and Menzies, N.W., 2011. Effect of fresh green waste and green waste compost on mineral nitrogen, nitrous oxide and carbon dioxide from a Vertisol. *Waste Management*, 31(8), pp.1720-1728.

Vitousek, P.M., D'antonio, C.M., Loope, L.L., Rejmanek, M. and Westbrooks, R., 1997. Introduced species: a significant component of human-caused global change. *New Zealand Journal of Ecology*, pp.1-16.

Wallenstein, M.D., McMahon, S.K. and Schimel, J.P., 2009. Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. *Global Change Biology*, 15(7), pp.1631-1639.

Wallenstein, M., Allison, S.D., Ernakovich, J., Steinweg, J.M. and Sinsabaugh, R., 2010. Controls on the temperature sensitivity of soil enzymes: a key driver of in situ enzyme activity rates. In: *Soil Enzymology*. Springer: Berlin, Heidelberg, pp.245-258

Williams, P.L., 1973. The validity of the application of simple kinetic analysis to heterogeneous microbial populations 1. *Limnology and Oceanography*, 18(1), pp.159-165.

Chapter 5

Sustainable urban agriculture using compost and an open-pollinated maize variety

Ulm, F., Avelar, D., Hobson, P., Penha-Lopes, G., Dias, T., Máguas, C. and Cruz, C., 2019. Sustainable urban agriculture using compost and an open-pollinated maize variety. *Journal of Cleaner Production*, 212, pp.622-629.

5.1 Abstract

Global urbanization leads to the loss of periurban farming land and increases dependency on distant agriculture systems. This provokes greenhouse gas emissions associated with transportation and storage while disconnecting nutrient cycles, as urban organic waste is not recycled into the agricultural system. Urban food production based on composted local biomass could reduce these problems, but currently used hybrid crops rely strongly on inorganic fertilizers. On the contrary, open-pollinated varieties were bred for productivity under organic fertilization, such as compost.

Hypothesising that open-pollinated varieties retain high nutritional value under low nutrient conditions, a commercial hybrid and a local open-pollinated variety of maize were cultivated in non-fertilized soil and under two compost applications: Municipal compost as high nutrient input or locally produced green waste compost and municipal compost mix, as medium nutrient input. Unfertilized plots exhibited low grain production (1.9 t ha^{-1}), but yields under green waste compost/municipal compost (6.1 t ha^{-1}) and municipal compost (7.8 t ha^{-1}) treatments were comparable to observations from maize under inorganic ferti-

lization. Contrary to the commercial variety, the open-pollinated variety exhibited higher grain micronutrient concentrations, e.g. 220% higher zinc concentrations and lower accumulation of heavy metals, e.g. 74% lower nickel concentrations. This variety-related effect was found in all treatments and was independent of soil micronutrient concentrations. In conclusion, both compost mixes were effective in increasing grain yield in both maize varieties. However, the open-pollinated variety produced grain with higher nutritional values in soil and all treatments, indicating it is potentially better suited for compost-based sustainable urban agriculture.

5.2 Introduction

Global society reached a turning point in 2007 when urban populations exceeded the population living on the countryside (United Nations, 2010), a trend which is expected to continue as cities become polycentric and new peri-urban centres emerge close to existing urban conurbations (Satterthwaite *et al.*, 2010). This development has far reaching consequences for the lives of people in the urban environment. In general, urbanization leads to improved living standards and life expectancy, however, it also becomes increasingly challenging to create resilient urban food supply systems, with subjects such as malnourishment as well as food and nutrition insecurity being pending issues in urban environments (Knorr *et al.*, 2018).

Peri-urban farming might be a viable option to produce food close to urban agglomerations and relieve some of the problems of urban nutrition, however, the rate of expansion of most cities worldwide exceeds urban population growth, which has intensified the competition for nearby agricultural land (Seto and Ramanakutty, 2016). The increasing distance between food production and urban consumption sites gives rise to a vast infrastructure needed to distribute and store food while increasing greenhouse gas emissions (GHG) and food waste (Bloem and de Pee, 2017). Another large part of urban waste is organic waste originating from urban green spaces such as gardens, parks and wastelands, which together constitute the largest source of municipal solid waste (Reyes-Torres *et al.*, 2018). This green waste is not only composed of pruning from planted species, but is also increasingly derived from

invasive plant species, which rapidly dominate invaded ecosystems and threaten native species found in urban landscapes (Alvey, 2006). Removing this biomass can decrease invasive species pressure, while providing an important source of biomass. However, as urban green waste has a low bulk volume and little economic value, it is commercially unappealing and expensive to collect and process, thus most often this waste ends up almost entirely in landfills on former agricultural land (Adhikari *et al.*, 2010), with further consequences for water quality and GHG emissions. A possible contribution to solve the issues of waste management and urban food security at the same time is to compost organic urban waste. This process also has great potential in diminishing GHG emissions, as compost can be used as soil amendment, increasing soil organic matter (SOM) concentrations while sequestering carbon (Bong *et al.*, 2017). Increased SOM concentrations additionally help to maintain soil structure and reduce nutrient leaching, and can turn degraded urban sites into productive farmland (Beniston *et al.*, 2016). Thus, municipal waste compost (MC) could help to create a clean, zero-waste system where resources are reused for urban and peri-urban farming (Lim *et al.*, 2016). While MCs often exhibit a high nutrient content (Cerdeira *et al.*, 2017), other frequently used composts, such as green waste compost (GWC) are poor in macronutrients essential for plant growth (Reyes-Torres *et al.*, 2018). Nevertheless, GWC has many beneficial aspects as an organic soil amendment, such as high recalcitrance, high C:N ratios as well as low heavy

metal pollution. Due to these characteristics, mixing GWC into contaminated urban soils can decrease heavy metal loads (Fitzstevens *et al.*, 2017). This could be of interest in urban agriculture, as high-nutrient urban composts, such as MC, often also exhibit high levels of contaminants and heavy metals, which accumulate along the food chain (Wei *et al.*, 2017). While there is substantial work available on mixing feedstock for GWC production with nutrient-rich material, such as manure, food waste or inorganic fertilizers in order to decrease composting time or improve compost quality itself (Reyes-Torres *et al.*, 2018), only recently GWC with subsequent fertilizer combinations were assayed for peri-urban food production, with promising results (Eldridge *et al.*, 2018). However, there are still many issues to address, for example, usage of compost with low nutrient content, such as GWC/MC mixes, can lead to yield reduction if used without chemical fertilizer on very nutrient demanding crops, such as tomato (Ribas-Agustí *et al.*, 2017). In some pedo-climatic conditions, the addition of MC might lead to lower yields compared to conventional farming, at least when considering short term compost application (Forte *et al.*, 2017). On the other hand, after an initial decrease in maize yield in the first year of application, pure GWC/MC treatments can perform better along time than conventional farming or even mixtures of GWC/MC with additional inorganic fertilizer (Bedada *et al.*, 2014). Also, while lower yields in organic agriculture working with compost might be inherent, there is also ample evidence that food produced using only organic amendments is more nutritious (Rahmann *et al.*, 2017). This is of increasing importance

for urban populations, as there remain serious deficiency problems for nutritionally essential micro-nutrients, even in heavily industrialized countries (FAO, 2013).

Even though micronutrient deficiency in urban centres remains a problem, genetic crop selection is still mainly aimed at increasing growth and yield in food crops, with an associated decrease in micronutrient and vitamin concentrations in the edible portion of the plant, the so-called “dilution effect” (Marles, 2017). This effect is long known and most noticeable in high yielding staple crops of global importance, such as maize, which accounts for 20% of the global food calories (Pixley and Bjarnason, 2002). Also, modern commercial food crops were bred to exploit an abundance of nutrients to produce high yielding growth (van Bueren *et al.*, 2011), while in contrast, many of the more traditional, open-pollinated varieties (OPV) were bred to maintain yields and nutrient concentrations also in low-input agricultural systems (Patto *et al.*, 2008). Thus, the biofortification (increase in nutritional value) of staple crops, such as maize, has become a pending issue and recently, OPVs were recognized for being valuable genetic sources for this undertaking (Puglisi *et al.*, 2018). Taking these observations about plant nutrition into account, research into increased recycling of nutrients from society (Röös *et al.*, 2018) and the selection of crop varieties accustomed to low-input farming (Tsvetkov *et al.*, 2018) are research goals of utmost importance to improve sustainability in (urban) agriculture.

With these major topics in mind, the work presented here joins nutrient recycling through composting with the prospect of OPVs of maize (*Zea mays*) as potentially biofortified

varieties. In the urban context of constrained space, high growth rates and yields are essential to turn food production relevant, however, to also be sustainable, plant nutrition needs to rely on locally available sources. In a novel approach both of these aspects were assessed simultaneously by growing a commercial hybrid variety and an OPV solely on MC and GWC as nutrient sources and subsequently measuring yield and mineral nutrition. The treatments consisted of either non-fertilized local soil or two types of nutrient applications: either MC alone as a high nutrient input, or mixed with GWC as a lower nutrient input treatment. Apart from exhibiting lower nutrient concentrations, it was also assumed that GWC might have lower heavy metal concentrations. As the GWC employed was derived from a local invasive species (*Acacia longifolia*), it represents, to our knowledge, the first test of compost from this feed- stock as an organic soil amendment for maize. Plant vegetative and grain yield as well as soil and grain micro and macronutrients were measured using inductively coupled plasma-optical emission spectroscopy as a state of the art methodology for ionomic profiling (Jaradat and Goldstein, 2018) and the data sets analysed using modern multivariate and univariate techniques. Putting forward the hypothesis that while the compost treatments would in general increase maize yield, it was also postulated that the OPV exhibits higher grain nutrient concentrations than the commercial variety, thereby putatively rendering it a more suitable option for this type of agriculture.

5.3 Materials and methods

5.3.1 Sample dates, setup and preparation

The study site is located within the Permaculture Living Laboratory (“PermaLab”) on the campus for Faculty of Science at the University of Lisbon (38°45′29.30″N, 9°9′30.40″W), and measures approximately 30 m by 10 m. It was divided into 16 smaller plots, each measuring 4 m². An automatic drip feed system (GREENTEC, AZUD, Spain) regulated equal water supply to each of the sub-plots throughout the growing period. Aside from the control plots, different mixtures of composts were applied superficially to the other plots and the top 20 cm of soil horizon mixed with the respective composts using a gasoline tiller (GM 105FQSTYLE, Chongqing Jiamu Machinery Co. Ltd, China). The MC used in this study was bought from the company ValorSul in Lisbon and is derived from food waste which is anaerobically digested for biogas production and then composted using wood chips as a bulking agent (ValorSul, 2018) (Class IIA from Decreto-Lei no 103/2015). GWC for the GWC/MC treatments was prepared on site from *Acacia longifolia* plant material (Ramos, 2016). In brief, plant material was harvested and ground mechanically into pieces < 10 cm and then composted in custom made bioreactors. The bioreactors were build from ROOFMATE_{TM} (DOW, 2018) extruded polystyrene foam insulation sheets of 5 cm thickness, which were joined using polyurethane foam adhesive spray and sealed with acrylic to maintain temperature and humidity constant. Compost was cured for 40 days in controlled mesophilic conditions (40 - 53°C),

keeping volumetric water content above 40%. Final mature composted material had: 1.5 N %, 48.0 C % and a C:N ratio of 34.3.

The extrapolated amount of compost applied in all cases was 2812.5 m³/ha, based on experiments from Brito *et al.* (2015), who used *Acacia longifolia* compost mixed with other substrates and soil as horticultural growth substrates (50% v/v). The MC treatments were prepared by adding 2812.5 m³/ha MC to the plots and the GWC/MC treatments were prepared by mixing in 937.5 m³/ha of mature GWC with 1875 m³/ha of MC before application. After compost application, the experimental plots were left to settle for one week and maize grain then sown in rows by hand to a depth of 1 cm, at a density of 5.5 plants/m² towards the end of June 2016. No biocides or mechanical clearing were applied during the course of the experiment and all plots received equal amounts of tap water by drip irrigation. At the end of the growing season (first week of October), plant height and amount of ears were determined and aboveground biomass of all plants was harvested and separated out in to stems, leaves, ear husks, cobs and grain. The material from each plant was air-dried at room temperature and weighed. To account for interference from air humidity, sub-samples were taken and dried in an oven at 60°C until constant weight to extrapolate total dry weight per plant. Plant height, number of ears and dry weight of plant and grain mass were treated as pseudo replica, pooled per plot and subsequently their mean was used for statistical analysis. If any plant was damaged by adverse conditions, like wind or animal interference,

etc. it was removed from analysis, however, there were never less than 6 plants pooled per plot. This way, the true replicates per plot and variety were: $n_{\text{Control}} = 5$; $n_{\text{MC}} = 5$; $n_{\text{GWC/MC}} = 3$. Nutrient analysis were done on pooled grains per variety per plot and to assess single kernel weight, kernels were grouped by variety and the weight of a single kernel calculated by extrapolating from weighing 36 batches of 10 kernels each.

5.3.2 Plant variety and phenology

The maize varieties used in this experiment were a commercial hybrid variety (SY Sincero, Syngenta, Portugal), termed “commercial variety” later in the text, and a traditional, regional OPV called “pata-de-porco multicolorido”, which describes its multi-coloured kernels and ear fasciation.

5.3.3 Soil collection, preparation and organic matter analysis

At the end of September 2016, 3 topsoil subsamples were taken from each plot to a depth of 10 cm from the surface and mixed thoroughly to obtain a pooled sample. The collected soil was sifted through a 2 mm sieve and then dried at 60°C until constant weight before being transferred to a muffle furnace (L3 Nabertherm, Lilienthal, Germany) for 6 h at 600°C to determine the soil organic matter. This process was followed by further exposure for 2 h at 950°C to determine the carbonate content on loss of ignition in accordance with the method described in Heiri et al. (2001). Finally, each sample was ground to produce a powder using a ball mill (Mixer Mill MM 400, Retsch, Germany) in preparation for isotopic and ionic analysis.

5.3.4 Iononics, total carbon, nitrogen and stable isotope analysis

Iononics values for each of the plants were determined using inductively coupled plasma optical emission spectrometry (ICP- OES; Thermo Elemental Iris Intrepid II XDL; Franklin, MA, USA) after a microwave assisted digestion with $\text{HNO}_3\text{:H}_2\text{O}_2$ (4:1, v:v) in the Iononics Service of the Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC, Spain) following ISO/IEC guidelines (UNE-EN ISO/IEC 17025, 2018).

Stable isotope ratio analyses were also measured at the Stable Isotopes and Instrumental Analysis Facility (SIIAF), Centre for Ecology, Evolution and Environmental Changes (cE3c), located in the Faculty of Sciences, University of Lisbon - Portugal. Values for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were determined for each sample using continuous flow isotope mass spectrometry (CF-IRMS), on a Sercon Hydra 20-22 (Sercon, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion (Preston and Owens, 1983). A delta Calculation was performed according to $\delta = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] * 1000$, where R is the ratio between the heavier and lighter isotopes. In the analysis, $\delta^{15}\text{N}_{\text{Air}}$ values refer to concentrations found in air, and $\delta^{13}\text{C}_{\text{VPDB}}$ values are referred to as PDB (Pee Dee Belemnite). The reference materials used were Sorghum Flour Standard OAS and Wheat Flour Standard OAS (Elemental Microanalysis, UK), for nitrogen and carbon isotope ratio (where $\delta^{15}\text{N}_{\text{Air}}$ (Sorghum Flour OAS) = $1.58 \pm 0.15\text{‰}$, $\delta^{15}\text{N}_{\text{Air}}$ (Wheat Flour OAS) = $2.85 \pm 0.17\text{‰}$, and $\delta^{13}\text{C}_{\text{VPDB}}$ (Sorghum Flour OAS) = $13.68 \pm 0.19\text{‰}$, $\delta^{13}\text{C}_{\text{VPDB}}$

(Wheat Flour OAS) = $27.21 \pm 0.13\text{‰}$) (Coleman and Meier-Augenstein, 2014). The level of predicted uncertainty in the observed value for the isotope ratio analysis was $\leq 0.1\text{‰}$ and calculated using the results from 6 to 9 replicates of secondary isotopic reference material (wheat flour, $\delta^{15}\text{N}_{\text{Air}} = 2.88 \pm 0.19\text{‰}$, $\delta^{13}\text{C}_{\text{PDB}} = 27.27 \pm 0.19\text{‰}$), which were interspersed among samples in every batch analysis. The major mass signals for N and C were used to calculate total N and C abundances, using Sorghum Flour Standard OAS and Wheat Flour Standard OAS, with 1.47% N, 46.26% C and 1.47% N, 39.53% C respectively, as elemental composition reference materials (Elemental Microanalysis, UK).

5.3.5 Statistical analysis

Plant traits (Height, Plant Weight, Grain Weight, Ears) were measured for each plant to provide a mean value calculated for all plants per plot. Stable isotope ratio and Ionic analyses were performed on aggregated sample data for each plot. Statistical analyses were performed with the package “stats” using version R 3.3.2 (R Core Team, 2016) and executed on RStudio (IDE version 1.0.136). Additional packages used were: “Hmisc”, “lawstat”, “lmttest” and “FactoMineR” (Lê *et al.*, 2008). Pairwise comparisons between groups were calculated using Pairwise Welch’s t-test with Bonferroni - Holm correction. If only two groups were compared, Welch’s t-test was used. Normality assumptions for group wise comparisons were verified using the Shapiro-Wilk Normality Test. If the normality assumption was violated, data was transformed to log or square root values. Linear regressions were performed after verifying assumptions using

the Breusch-Pagan Test for homoscedasticity and the Shapiro-Wilk Normality Test on the regression model residuals. **Figure 5.3** is a network plot created from a spearman correlation matrix. **Figure 5.4** is based on an RV coefficient matrix, generated by using the package FactoMineR to highlight relationships between groups of variables. For multivariate explorative data analysis of grain and plant traits, a multiple factor analysis (MFA) was employed, using plant traits, grain nutrients and the two factors treatment type and plant variety as input (**figure 5.5**).

5.4 Results and discussion

5.4.1 Soil changes

The topsoil conditions in all the study plots were significantly altered by the compost addition (**table 5.1**). Apart from SOM, the quantities of total C and N were both ca. 10-fold higher in the treatment plots than in the control. For most macro- and micronutrients, soil amended with MC exhibited higher nutrient

levels than with GWC/MC, whilst the control soil plots recorded the lowest values for all nutrients except for K and Mn, where no significant difference was found. The original soil in the study plot had lower than average levels of N and S for the city (0.31% and 0.05%, respectively) (Costa *et al.*, 2012), while after the compost treatment macro- and micronutrient

Table 5.1 Topsoil parameters (< 20 cm) of untreated soil (control), soil with municipal compost (MC) and soil with a mix of green waste compost and municipal compost (GWC/MC).

Numbers depict means ($n_{\text{Control}} = 5$; $n_{\text{MC}} = 5$; $n_{\text{GWC/MC}} = 3$) with standard errors in parentheses. Letters depict significant differences (pairwise Welch's t-test with holm correction; $p < 0.05$, values were square root transformed to conform with normality assumption) significant differences between groups are shown in bold.

		GWC/MC	MC	Control
General characteristics	% SOM	16.99 (3.44)^a	23.05 (2.07)^a	5.69 (0.21)^b
	% Carbonate	3.55 (0.43)^a	4.41 (0.34)^a	2.06 (0.16)^b
	$\delta^{15}\text{N}$	10.7 (0.31)^a	9.98 (0.35)^a	5.52 (0.11)^b
	$\delta^{13}\text{C}$	-24.13 (0.09)^a	-23.64 (0.2)^a	-20.14 (1.01)^b
	% N	0.9 (0.15)^a	1 (0.08)^a	0.12 (0.02)^b
Macronutrient concentrations mg g ⁻¹	% C	9.27 (1.7)^a	11.6 (0.72)^a	1.64 (0.18)^b
	P	5.86 (1.48)^b	9.72 (1.17)^a	0.31 (0.02)^c
	K	3.79 (0.73)	4.22 (0.48)	4.07 (0.28)
	Ca	34.77 (6.75)^b	49.57 (4.94)^a	12.22 (0.76)^c
	S	1.64 (0.37)^b	2.43 (0.25)^a	0.3 (0.01)^c
	Mg	3.57 (0.8)^{ab}	4.57 (0.29)^a	2.67 (0.26)^b
	Na	0.71 (0.17)^{ab}	1.16 (0.22)^a	0.26 (0.03)^b
Micronutrient and heavy metal concentrations µg g ⁻¹	B	11.15 (2.41)^{ab}	14.35 (1.05)^a	7.33 (0.48)^b
	Mn	145.96 (25.79)	174.48 (5.6)	180.61 (12.55)
	Zn	119.09 (27.06)^b	172.91 (14.9)^a	42.33 (1.41)^c
	Fe	10,895.99 (1381.13)^a	9,965.15 (725.88)^a	15,749.67 (685.98)^b
	Cu	29.66 (5.91)^b	41.62 (2.76)^a	16.33 (1.39)^c
	Mo	0.44 (0.09)^a	0.53 (0.15)^a	0.08 (0.05)^b
	Ni	10.19 (1.33)	11.08 (0.42)	12.76 (0.81)
	Cd	0.37 (0.1)^{ab}	0.55 (0.05)^a	0.28 (0.02)^b
	Pb	19.32 (2.4)	19.59 (0.69)	22.21 (0.91)
	Cr	44.31 (2.68)	44.12 (3.02)	43.47 (3.48)
	As	0.33 (0.11)	0.95 (0.34)	0.16 (0.14)

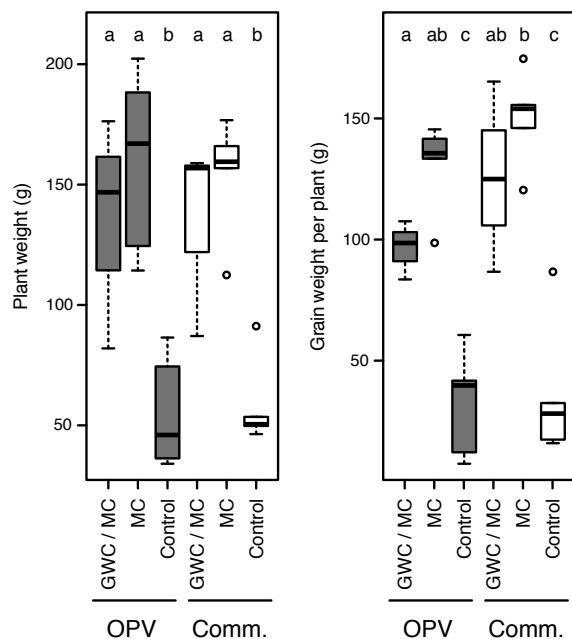


Figure 5.1 Differences in plant and grain weight of both corn varieties in all treatments.

Boxplots describing the differences in vegetative plant weight (left) and grain weight (right) of both corn varieties (open-pollinated and commercial) in all treatments (per variety: $n_{\text{Control}} = 5$; $n_{\text{MC}} = 5$; $n_{\text{GWC/MC}} = 3$). Letters depict significant differences (pairwise Welch's t-test with holm correction; $p < 0.05$). GWC = Green Waste Compost; MC = Municipal Compost. OPV = open-pollinated variety, Comm. = commercial variety.

concentrations far exceeded the mean concentrations reported for most Lisbon soils (Costa *et al.*, 2012). Urban environments are often viewed as hostile to food crop production because of unnaturally high levels of heavy metals; particularly bulk lead (Fitzstevens *et al.*, 2017). However, readings for heavy metals were unaltered by the treatments with the exception of Cd, which in the MC treatment showed levels twice as high as in the control plots, while for the plots treated with GWC/MC, there was a moderate increase in Cd. In any case, heavy metal concentrations well below the lower guideline values considered to be of risk to the ecology of soils and even for Cd, where soil with MC additions showed increased values, ca. half of the threshold value of 1 mg Cd per g soil (Toth *et al.*, 2016).

In the case of macronutrients, the largest dif-

ferences between treated and control plots were observed for P levels, which were 32 times higher under MC treatment, and 20 times higher under GWC/ MC treatment. Most of the readings for macro- and micronutrient levels registered between 1.5 and 3 times higher in the MC treatment, with the exception of Mo, which was 7 times higher in the MC plots, and 6-fold higher in the plots subjected to GWC/ MC treatment. However, even though GWC/ MC and MC treatments were effective in increasing Mo concentrations, the values recorded here were nevertheless lower than Lisbon average values of 1.67 mg g⁻¹ (Costa *et al.*, 2012) and at the plant growth limiting end of soil Mo concentrations (Marschner, 2012).

5.4.2 Effects on plant biomass and nitrogen

In all experimental plots treated with compost the growth of maize plants and the accumulation of biomass were significantly higher than they were in the control sites, irrespective of the variety used (**figure 5.1, left**). Similarly, grain yield was significantly higher in treated plots (**figure 5.1, right**), although, the commercial variety grown in MC plots yielded higher grain counts than the OPV grown in GWC/MC plots. Based on the values displayed in **figure 5.1** (grain yield per plant), the average grain yield for the OPV of maize was extrapolated to be 1.8 t ha⁻¹ in the control plots; 5.3 t ha⁻¹ in the GWC/ MC plots; and 7.2 t ha⁻¹ for soil treated with MC. For the commercial variety, the average grain yield was calculated at 2 t ha⁻¹ in the control plots; 6.9 t ha⁻¹ in the GWC/MC plots; and 8.3 t ha⁻¹ in the MC plots. Thus, both maize varieties grown with compost compete favourably in grain yield with

Table 5.2 Nutrient concentrations in maize grain of plants growing in soil without treatment (Control), soil with municipal compost (MC) and soil with a compost-mix (GWC/MC) of green waste compost (GWC) and MC. All values are given in mg g⁻¹, values in bold show significant differences between open-pollinated and commercial variety. Numbers depict means (per variety: n_{Control} = 5; n_{MC} = 5; n_{GWC/MC} = 3) with standard errors in parentheses. Asterisks depict p-values (* = p < 0.05, ** = p < 0.01, *** = p < 0.001; Welch's t-test, values were square root transformed to conform with normality assumption). OPV = open-pollinated variety, Comm = commercial variety. Significant differences between groups are shown in bold.

	GWC/MC		MC		Control	
	OPV	Comm.	OPV	Comm.	OPV	Comm.
N	15,333.33 (1,666.67)	9,666.67 (333.33)	16,200 (969.54)	12,200 (1,019.80)*	14,200 (1,240.97)	9,800 (374.17)*
P	4,411.80 (78.59)	2,740.47 (202.43)*	4,455.05 (99.36)	2,567.32 (72.15)***	4,028.44 (305.09)	2,747.81 (147.45)**
K	5,396.02 (192.22)	3,566.51 (189.06)**	5,345.69 (111.04)	3,679.58 (208.68)**	5,253.05 (248.11)	3,688.55 (199.39)**
S	1,105.02 (89.76)	713.64 (39.16)*	1,174.91 (20.41)	758.48 (30.82)***	1,002.36 (71.97)	804.44 (46.53)
Mg	1,846.34 (97.23)	1,015.90 (101.56)**	1,907.84 (60.22)	939.79 (39.24)***	1,582.74 (160.11)	1,046.24 (54.63)*
Ca	75.81 (6.41)	45.76 (8.68)	79.71 (6.07)	59.78 (7.05)	97.79 (9.29)	90.07 (9.80)
B	2.47 (0.14)	1.76 (0.04)*	2.49 (0.22)	1.67 (0.19)*	2.39 (0.18)	1.89 (0.08)*
Mn	19.93 (1.31)	10.89 (0.80)**	22.26 (1.02)	10.84 (0.41)***	19.16 (1.58)	12.34 (0.45)**
Zn	53.36 (3.77)	23.28 (2.15)**	54.64 (1.75)	23.22 (1.14)***	50.81 (5.16)	26.47 (1.27)**
Fe	30.90 (4.89)	23.74 (2.51)	39.68 (2.79)	29.67 (7.00)	31.88 (2.15)	21.94 (3.20)*
Cu	3.00 (0.04)	1.57 (0.07)**	3.45 (0.08)	1.66 (0.11)***	3.20 (0.32)	1.76 (0.16)**
Mo	0.24 (0.11)	0.01 (0.01)	0.25 (0.08)	0.01 (0.01)*	0.23 (0.08)	0.00 (0.00)**
Ni	0.07 (0.05)	0.24 (0.14)	0.02 (0.01)	0.20 (0.07)*	0.20 (0.05)	0.51 (0.06)*

industrially produced transgenic or non-transgenic commercial hybrid varieties in Portugal (Skevas *et al.*, 2010). By extrapolating from the yield data, ca. 22 m² of compost-fed urban ground would be sufficient to produce enough maize in the period of 3 months to support one individual for the year, leaving the rest of the year to grow other crops.

Interestingly, while there were marked differences in plant weight and grain yield between treatments (F = 26.71, p < 0.001 for GWC/MC plots; and F = 50.58, p < 0.001 for the

MC plots), there was no clear evidence of significant differences in N concentrations in the grain between treated plots (F = 2.86, p = 0.081), but there was a significant difference in levels of N between the two varieties (F = 29.58, p < 0.001). Grain from the OPV yielded higher N concentrations in both control as well as all treated plots (**table 5.2**), indicating higher protein content in this variety. The mean difference observed here (1.4 times more N in the OPV vs. the commercial variety) is in accordance with results found for oth-

er Portuguese OPVs, which reported up to 1.3 times higher protein content in OPVs, compared to hybrid varieties (Brites *et al.*, 2010). This effect might be due to higher N use efficiency inherent to this variety, as OPVs of maize might be more resource efficient under low nutrient conditions (Omondi *et al.*, 2014) and accumulate more protein (Flint-Garcia, 2017). Similar to the pattern observed for N concentrations, K and P concentrations did not change in response to the treatments ($F = 0.0258$, $p = 0.974$ and $F = 0.5191$, $p = 0.603$, respectively), but were significantly higher in the OPV kernels in all treatments ($F = 100.64$, $p < 0.001$ and $F = 116.1389$, $p < 0.001$, respectively). While mean K concentrations found in the commercial variety (3645 mg g^{-1}) are below values found in OPVs in another study (3745 mg g^{-1} ; Jaradat and Goldstein, 2018), the OPV observed here exhibits substantially higher K concentrations (5332 mg g^{-1}). On the contrary, the P values observed in the commercial variety are lower (2685 mg g^{-1}) than what was observed in OPVs in the study mentioned above (3362 mg g^{-1} ; Jaradat and Goldstein, 2018), while they are higher than in the OPV observed in this study (4298 mg g^{-1}). In contrast to K, where an increased concentration in OPVs kernels is a beneficial trait, higher P values can be potentially problematic if P is stored mainly as phytic acid. The ratio of free P in comparison to phytic acid was not measured, however, it is known from other OPVs that they can potentially exhibit high free P and low phytic acid levels (Puglisi *et al.*, 2018v), rendering them more suitable as P source for human consumption.

While the OPV was apparently more efficient in nutrient acquisition, resource efficiency

was not reflected in grain weights, with significantly lower weights (Welch's t-test, $p < 0.001$), for the OPV with $0.312 \text{ g} (\pm 0.004 \text{ SE})$, compared to the commercial variety, which yielded $0.347 \text{ g} (\pm 0.005 \text{ SE})$ per grain. This is contrary to earlier reports indicating higher kernel weights for OPVs compared to hybrid varieties, however kernel weights were in general high in comparison to values observed by other authors, ranging around $0.26\text{--}0.28 \text{ g}$ (Flint-Garcia, 2017). On the other hand, the increased grain weight in the commercial variety could be related to the dilution effect, which is known to occur in maize (Marles, 2017). While in both varieties grain yield was clearly connected with the compost treatments (**figure 5.1**), only the grain of the commercial maize variety exhibited a linear, positive correlation between grain N concentrations with SOM levels (**figure 5.2**). Thus, both varieties respond to fertilization with increased grain yield, however, the commercial variety also

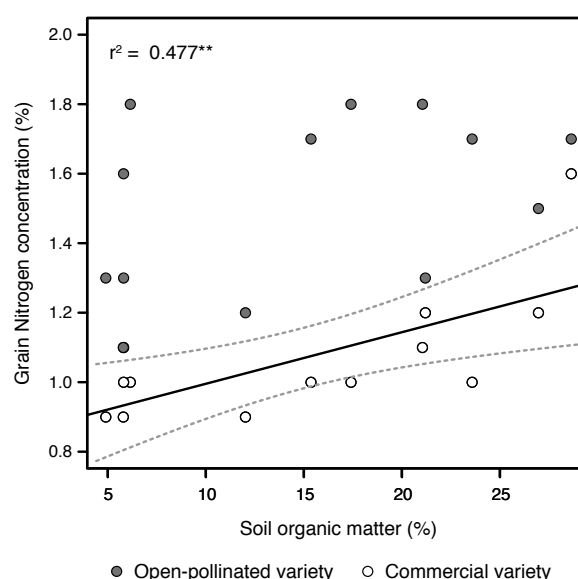


Figure 5.2 Pearson correlation between grain nitrogen (N) content and soil organic matter (OM). Dark grey dots depict the open-pollinated variety, white dots the commercial variety. Only the commercial variety shows a significant correlation between soil OM and grain N concentrations (black line, 95% confidence interval and r^2 shown).

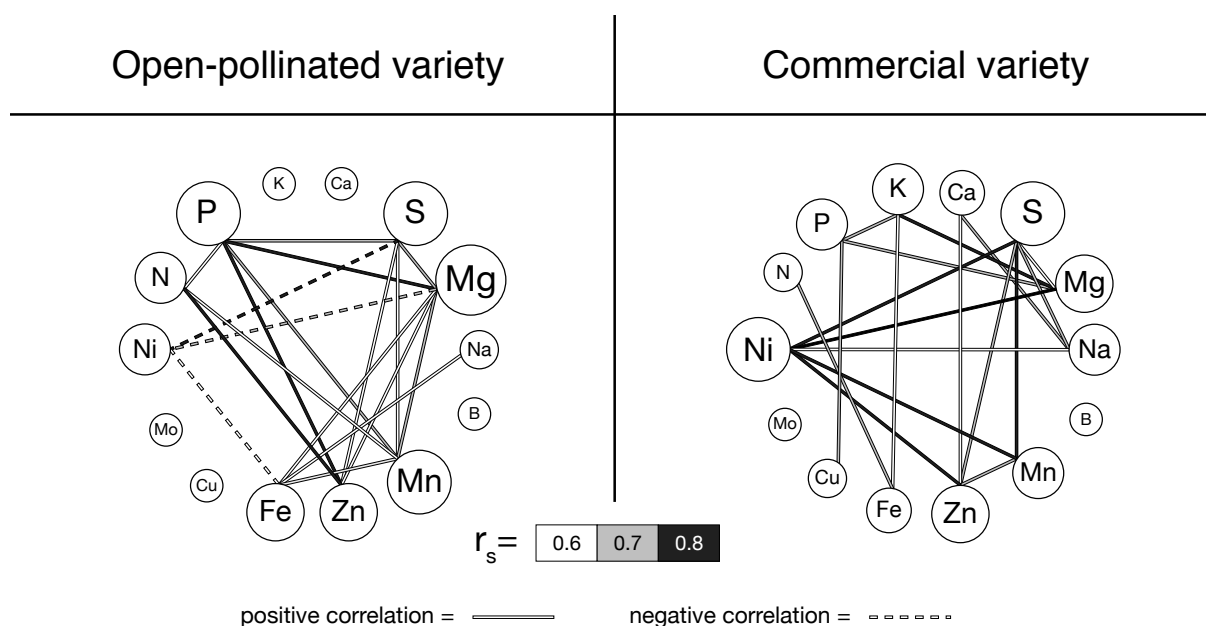


Figure 5.3 Nutrient Network between all macro- and micronutrients found in the maize kernels. Nutrient Network, based on a matrix of spearman correlations (r_s) between all macro- and micronutrients found in the maize kernels (dotted lines indicate negative correlation, full lines indicate positive correlations). White, grey or black lines indicate the strength of the relationship (0.6, 0.7 and 0.8, respectively), while the sphere sizes indicate the amount of existing relationships (min = 0; max = 6), basis for the nutrient network was $n = 13$ for each variety.

increases nutritional value with increased fertilization, while the OPV accumulates N largely independent of fertilization. From a nutritional standpoint, the OPV is therefore indicated as a better food source also in nutrient-poor agricultural regimes.

5.4.3 Grain micronutrient concentrations in commercial and open-pollinated varieties

The status of other nutrients recorded in grain samples was variable between treatments and maize varieties (Table 5.2). Apart from Ca, Ni and S, all other recorded nutrients were observed to be higher in the OPV growing under control conditions and also in the MC plots, with the exception of Ca and Fe. The results for the GWC/ MC plots indicated the main nutrients with the exception of Ca, Fe, Mo and Ni were higher in the OPV of maize. As maize is also a source of essential minerals and the average consumption of maize per capita in Por-

tugal is 48 g day^{-1} (FAOSTAT, 2013), this portion would provide 52% of females and 36% of males with their daily requirements of Zn if open-pollinated varieties were selected for (FAO and WHO, 2005). Current biofortification goals in breeding programs set the target goals for Zn and Fe concentrations in kernels at 33 and 52 mg g^{-1} , respectively (Hindu *et al.*, 2018), thus, kernels of the OPV even under unfertilized conditions achieve about 155% and 62%, respectively, of these targets. In terms of trace elements, the average consumption of OPV maize would furthermore provide 35% of daily needs of Mo (34 mg) (Food and Nutrition Board, Institute of Medicine, 2000). In contrast, the commercial variety would yield less than 2% of daily requirements. What is more, heavy metals did not accumulate in the grain of the OPV, and levels for Ni recorded in samples were around 0.51 mg g^{-1} , which would equate to 13.5% of the recommended daily threshold limit for Ni of $2.8 \text{ mg Ni kg}^{-1}$

body weight (ESFA, 2015) in an average diet of maize. An analysis using a network plot and Spearman's correlation matrix revealed a relationship between various macro- and micronutrients (**figure 5.3**). For instance, in the kernels of the OPV, macronutrients such as N, P, S and Mg were positively correlated with the micronutrients Mn and Zn, while Ni was negatively correlated with Fe, Mg and S. On the contrary, in the commercial variety, Ni was positively correlated with other nutrients, such as S, Mg, Na, Mn and Zn. Interestingly, in both varieties, no correlation was found between Zn and Fe, which is in accordance with Indian varieties, but in contrast to observations from African cultivars (Akinwale *et al.*, 2016). The

positive correlation between P and various micronutrients in the OPV was also found in several maize varieties selected for biofortified crops, potentially indicating a co-selection of traits relevant for higher kernel nutrient accumulation (Gu *et al.*, 2015). Contrary to these positive correlations, the correlations between S and Mg with Ni are negative in the OPV, while being highly positive in the commercial variety. This is potentially problematic, as any accumulation of micronutrients in this variety would be accompanied by an increase in Ni.

While RV coefficients (**figure 5.4**) suggest a strong relationship between soil micro- and macronutrient concentrations and grain production in both varieties, they also suggest that in the OPV, the micronutrient concentrations appear to be linked to plant traits, whereas, in the commercial variety these concentrations were more closely associated with soil micronutrient concentrations. These results could suggest the selective breeding strategies for commercial maize favour nutrient up-take and grain yield at the expense of other traits (van Bueren *et al.*, 2011). Thus, when growing these varieties under conditions of slow nutrient release, the genetic traits of the plant that dictate its eco-physiology are important considerations in strategies for sustainable urban agriculture. For example, the differences observed in our trials could be due to higher arbuscular mycorrhizal fungi (AMF) colonization rates in the OPV, which has been shown in other OPVs grown alongside commercial hybrids (Hess *et al.*, 2005). If this were the case, it would also explain the observed differences between the two varieties in the take-up of Ni. The commercial variety accumulated larger concentrations of this metal unlike its

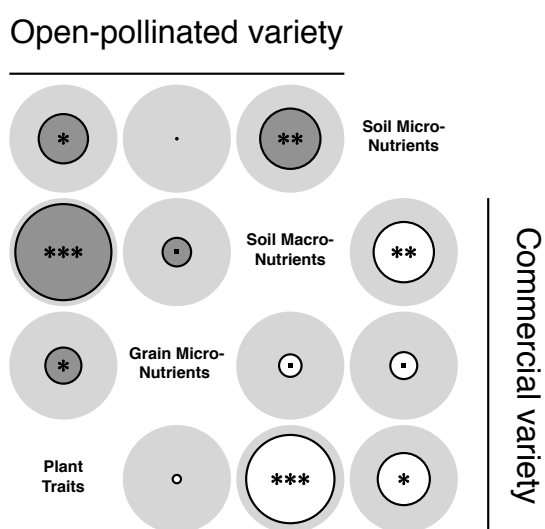


Figure 5.4 RV-coefficient matrix of data matrices used in this work.

Schematic describing the relationships between two data matrices each, sphere size shows similarity (RV-coefficients), with the light grey spheres indicating a similarity of 100%. Dark grey spheres indicate open-pollinated variety, white spheres commercial variety. Three asterisks: $p < 0.001$, two asterisks: $p < 0.01$, one asterisk: $p < 0.05$, square: $p < 0.1$, $n = 13$ per variety.

Plant Traits = Plant Height; Number of Ears; Grain weight; Vegetative Plant Weight; total grain C, N and P.

Soil Macronutrients = Soil organic matter (SOM), soil carbonates, total C, N, P, K, Ca, S, Mg.

Soil Micronutrients = Na, B, Mn, Zn, Fe, Cu, Mo, Ni, Cd, Pb, Cr.

Grain Micronutrients = Concentration of B, Mn, Zn, Fe, Cu, Mo, Ni, Mg, Ca, S, Na, K, Al in grain.

open-pollinated counter-part (**table 5.2, figure 5.3**). While AMF colonization was not measured, this would be an interesting field for further research, as changes in Ni accumulation has been reported after AMF colonization (Ramírez-Flores *et al.*, 2017).

A multiple factor analysis (MFA) using data on grain nutrient concentrations and plant traits (**figure 5.5, left**) revealed again a strong distinction between the OPV and commercial variety. The first dimension (X axis), explaining 41% of the total variance separated OPV and commercial variety, while the second dimension (Y axis), explaining 28% of the total variance, separated the different experimental treatments, in which the control plots were distinct from the two treatments. This orthogonal statistical response further under-

lines that variety effect and treatment effect were independent, with varieties being clearly separated by grain nutrient content (52%) as well as C, N and P concentration and the number of ears per plant (46%) (**figure 5.5, right**). Accordingly, the nutrient status of the grain is linked primarily to plant variety, while indicators of plant productivity, such as plant height and weight as well as grain weight per plant, are mainly related to the treatments applied. As development of multi-nutrient rich strains of maize is of utmost importance for the sustainable nutrition of the global population (Jaradat *et al.*, 2018), this pattern underlines the recently proposed selection of OPVs as a good way forward to achieve this feat (Puglisi *et al.*, 2018). Also, the clear, positive effect of both compost types on the yield of both

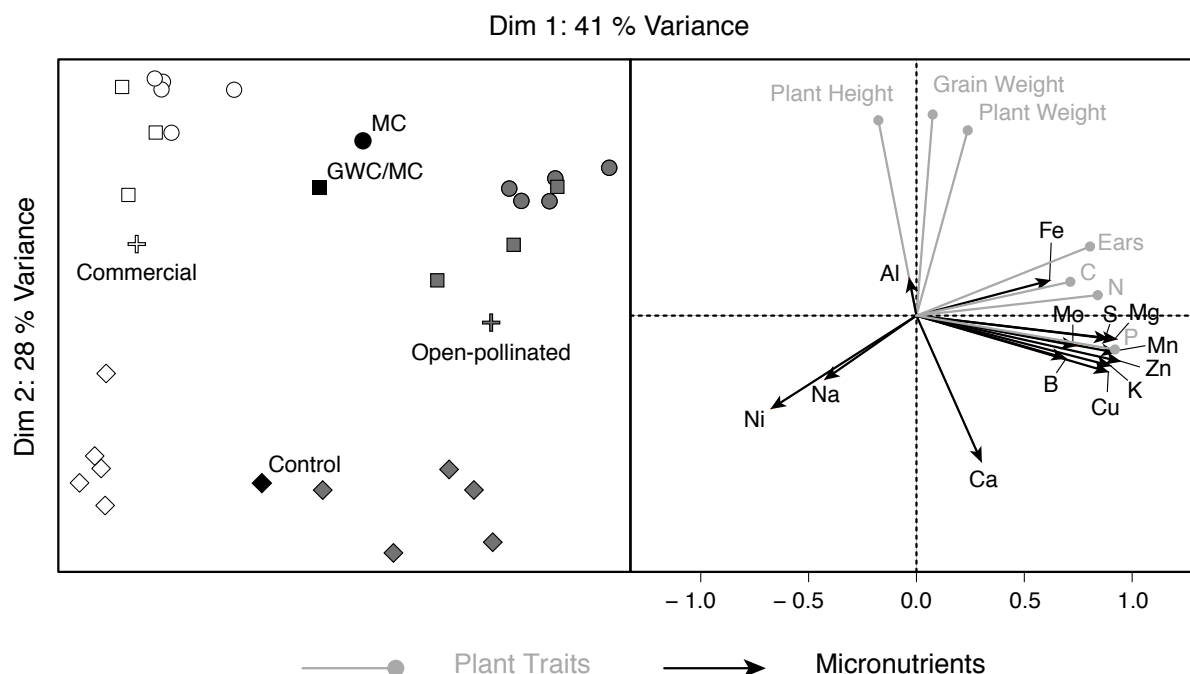


Figure 5.5 Multiple factor analysis (MFA) of variables related with the maize plants ($n = 13$ per plant variety).

The three groups used in this MFA were:

Grain micronutrient content = B, Mn, Zn, Fe, Cu, Mo, Ni, Mg, Ca, S, Na, K, Al

Plant traits = plant height, plant weight, grain weight, number of ears, total C, N, P Factors = treatments, varieties

Left: Individual factor map, including the centroids of the varieties (crosses), the individual data points of open-pollinated (grey) and commercial (white) maize varieties and the treatment centroids in black (sphere = municipal compost, square = municipal compost and green waste mix, diamond = untreated soil)

Right: Correlation cycle of all variables used.

maize varieties measured here, contributes to evidence from recently published work on the feasibility of using GWC in periurban farming (Eldrige *et al.*, 2018). Lastly, as the GWC used in this project was derived from a locally invasive plant (*A. longifolia*) and had no adverse effects on maize growth, e.g. similar responses than MC, these results add to recent work about its possible usage in agriculture or horticulture (Brito *et al.*, 2015).

5.4.4 Conclusions

This study demonstrated that it is possible to cultivate a staple food crop (*Zea Mays*) with adequate yields (up to 8.3 t ha⁻¹) in an urban setting, using only MC and GWC from nearby sources. As fertilization was based solely on soil amendments from composted food and green waste, this helps to achieve sustainable urban consumption patterns by recycling organic waste while increasing soil organic matter (up to 4 fold), thus mitigating GHG emissions. Furthermore, comparing an OPV and a commercial maize variety, it was found that the former exhibits higher nutrient use efficiency and increased macro- and micronutrient concentrations without accumulating heavy metals. It was found that this effect was variety and not treatment dependent, which points to OPVs as potentially interesting candidates for biofortified staple crops. In conclusion, this work contributes to a growing body of scientific evidence supporting alternative methods of intensive sustainable farming that can be adapted to urban landscapes.

Acknowledgements

We would like to thank the Faculdade de Ciencias da Universidade de Lisboa for the permission to set up the plots on the faculties' campus. Also, we would like to thank the association “Colher para Semear” (<https://colherparasemear.wordpress.com/>) for providing the open-pollinated seeds, as well the start-up 2AdaPt (<http://2adapt.pt>) for logistic support and all volunteers and guardians of the HortaFCUL permaculture project (<http://hortaful.wixsite.com/home>) for their help in setting up and maintaining the plots. We also would like to thank Fundação para a Ciência e a Tecnologia for supporting the PhD grant PD/BD/ 106061/2015 of FU, PhD grant PD/BD/52595/2014 of DA, as well as FCT Investigator contract (IF/00940/2015) of GPL.

5.5 References

- Adhikari, B.K., Trémier, A., Martinez, J. and Barrington, S., 2010.** Home and community composting for on-site treatment of urban organic waste: perspective for Europe and Canada. *Waste Management & Research*, 28(11), pp.1039-1053.
- Akinwale, R.O. and Adewopo, O.A., 2016.** Grain iron and zinc concentrations and their relationship with selected agronomic traits in early and extra-early maize. *Journal of Crop Improvement*, 30(6), pp.641-656.
- Alvey, A.A., 2006.** Promoting and preserving biodiversity in the urban forest. *Urban Forestry & Urban Greening*, 5(4), pp.195-201.
- Bedada, W., Karlton, E., Lemenih, M. and Tolera, M., 2014.** Long-term addition of compost and NP fertilizer increases crop yield and improves soil quality in experiments on smallholder farms. *Agriculture, Ecosystems & Environment*, 195, pp.193-201.
- Beniston, J.W., Lal, R. and Mercer, K.L., 2016.** Assessing and managing soil quality for urban agriculture in a degraded vacant lot soil. *Land Degradation & Development*, 27(4), pp.996-1006.
- Bloem, S. and de Pee, S., 2017.** Developing approaches to achieve adequate nutrition among urban populations requires an understanding of urban development. *Global Food Security*, 12, pp.80-88.
- Bong, C.P.C., Lim, L.Y., Ho, W.S., Lim, J.S., Klemeš, J.J., Towprayoon, S., Ho, C.S. and Lee, C.T., 2017.** A review on the global warming potential of cleaner composting and mitigation strategies. *Journal of Cleaner Production*, 146, pp.149-157.
- Brites, C., Trigo, M.J., Santos, C., Collar, C. and Rosell, C.M., 2010.** Maize-based gluten-free bread: influence of processing parameters on sensory and instrumental quality. *Food and Bioprocess Technology*, 3(5), pp.707-715.
- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S.R., 2015.** Co-composting of invasive *Acacia longifolia* with pine bark for horticultural use. *Environmental Technology*, 36(13), pp.1632-1642.
- Coleman, M. and Meier-Augenstein, W., 2014.** Ignoring IUPAC guidelines for measurement and reporting of stable isotope abundance values affects us all. *Rapid Communications in Mass Spectrometry*, 28(17), pp.1953-1955.
- Costa, C., Reis, A.P., Da Silva, E.F., Rocha, F., Patinha, C., Dias, A.C., Sequeira, C. and Terroso, D., 2012.** Assessing the control exerted by soil mineralogy in the fixation of potentially harmful elements in the urban soils of Lisbon, Portugal. *Environmental Earth Sciences*, 65(4), pp.1133-1145.
- Dow Chemical., 2018.** http://msdssearch.dow.com/PublishedLiteratureDOWCOM/dh_098b/0901b8038098b998.pdf?filepath=1/4styrofoam/pdfs/noreg/291-42625.pdf&fromPage=1/4GetDoc.
- EFSA Panel on Contaminants in the Food Chain (CONTAM)., 2015.** Scientific opinion on the risks to public health related to the presence of nickel in food and drinking water. *EFSA Journal*, 13(2), p.4002.
- Eldridge, S.M., Chan, K.Y., Donovan, N.J., Saleh, F., Orr, L. and Barchia, I., 2018.** Agronomic and economic benefits of green-waste compost for peri-urban vegetable production: implications for food security. *Nutrient Cycling in Agroecosystems*, 111(2-3), pp.155-173.
- Fitzstevens, M.G., Sharp, R.M. and Brabander, D.J., 2017.** Biogeochemical characterization of municipal compost to support urban agriculture and limit childhood lead exposure from resuspended urban soils. *Elementa Science of the Anthropocene*, 5.
- Flint-Garcia, S.A., 2017.** Kernel evolution: from teosinte to maize. In: *Maize Kernel Development*. CABI, Oxfordshire, pp. 1-15.
- Food and Agriculture Organization of the United Nations., 2013.** The state of food and agriculture. Rome.
- Food and Agriculture Organization of the United Nations and World Health Organization., 2005.** Vitamin and mineral requirements in human nutrition.
- Food and Nutrition Board, Institute of Medicine., 2000.** Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium and Zinc. National Academies Press. Food and Nutrition Board, Institute of Medicine, Washington (D.C.).
- Forte, A., Fagnano, M. and Fierro, A., 2017.** Potential role of compost and green manure amendment to mitigate soil GHGs emissions in Mediterranean drip irrigated maize production systems. *Journal of Environmental Management*, 192, pp.68-78.
- Gu, R., Chen, F., Liu, B., Wang, X., Liu, J., Li, P., Pan, Q., Pace, J., Soomro, A.A., Lübberstedt, T. and Mi, G., 2015.** Comprehensive phenotypic analysis and quantitative trait locus identification for grain mineral concentration, content, and yield in maize (*Zea mays* L.). *Theoretical and Applied Genetics*, 128(9), pp.1777-1789.
- Heiri, O., Lotter, A.F. and Lemcke, G., 2001.** Loss on ignition as a method for estimating organic and carbonate content in sediments: reproducibility and comparability of results. *Journal of Paleolimnology*, 25(1), pp.101-110.

- Hess, J.L., Shiffler, A.K. and Jolley, V.D., 2005.** Survey of mycorrhizal colonization in native, open-pollinated, and introduced hybrid maize in villages of Chiquimula, Guatemala. *Journal of Plant Nutrition*, 28(10), pp.1843-1852.
- Hindu, V., Palacios-Rojas, N., Babu, R., Suwarno, W.B., Rashid, Z., Usha, R., Saykhedkar, G.R. and Nair, S.K., 2018.** Identification and validation of genomic regions influencing kernel zinc and iron in maize. *Theoretical and Applied Genetics*, pp.1-15.
- Jaradat, A.A., Goldstein, W., 2018.** Diversity of maize kernels from a breeding program for protein quality III: Ionome profiling. *Agronomy* 8, 9.
- Knorr, D., Khoo, C.S.H. and Augustin, M.A., 2018.** Food for an Urban Planet: Challenges and Research Opportunities. *Frontiers in Nutrition*, 4, p.73.
- Lê, S., Josse, J. and Husson, F., 2008.** FactoMineR: an R package for multivariate analysis. *Journal of Statistical Software*, 25(1), pp.1-18.
- Lim, S.L., Lee, L.H. and Wu, T.Y., 2016.** Sustainability of using composting and vermicomposting technologies for organic solid waste biotransformation: recent overview, greenhouse gases emissions and economic analysis. *Journal of Cleaner Production*, 111, pp.262-278.
- Marles, R.J., 2017.** Mineral nutrient composition of vegetables, fruits and grains: The context of reports of apparent historical declines. *Journal of Food Composition and Analysis*, 56, pp.93-103.
- Marschner, H., 2012.** Marschner's Mineral Nutrition of Higher Plants. Elsevier, Amsterdam. The Netherlands.
- Omondi, E.C., Norton, J.B. and Ashilenje, D.S., 2014.** Performance of a local open pollinated maize variety and a common hybrid variety under intensive small-scale farming practices. *African Journal of Agricultural Research*, 9(11), pp.950-955.
- Patto, M.C.V., Moreira, P.M., Almeida, N., Satovic, Z. and Pego, S., 2008.** Genetic diversity evolution through participatory maize breeding in Portugal. *Euphytica*, 161(1-2), pp.283-291.
- Pixley, K.V. and Bjarnason, M.S., 2002.** Stability of grain yield, endosperm modification, and protein quality of hybrid and open-pollinated quality protein maize (QPM) cultivars. *Crop Science*, 42(6), pp.1882-1890.
- Preston, T. and Owens, N.J., 1983.** Interfacing an automatic elemental analyser with an isotope ratio mass spectrometer: the potential for fully automated total nitrogen and nitrogen-15 analysis. *Analyst*, 108(1289), pp.971-977.
- Puglisi, D., Landoni, M., Cassani, E., Toschi, I., Lucchini, G., Cesari, V., Borlini, G., Scapin, A. and Pilu, R., 2018.** Traditional farmers' varieties: a valuable source of genetic variability for biofortification programs. *Maydica*, 63(2), p.10.
- R Core Team., 2016.** R: a Language and Environment for Statistical Computing. R. Foundation for Statistical Computing, Vienna, Austria.
- Rahmann, G., Ardakani, M.R., Bàrberi, P., Boehm, H., Canali, S., Chander, M., David, W., Dengel, L., Erisman, J.W., Galvis-Martinez, A.C. and Hamm, U., 2017.** Organic Agriculture 3.0 is innovation with research. *Organic Agriculture*, 7(3), pp.169-197.
- Ramírez-Flores, M.R., Rellán-Álvarez, R., Wozniak, B., Gebreselassie, M.N., Jakobsen, I., Olalde-Portugal, V., Baxter, I., Paszkowski, U. and Sawers, R.J., 2017.** Co-ordinated Changes in the Accumulation of Metal Ions in Maize (*Zea mays ssp. mays* L.) in Response to Inoculation with the Arbuscular Mycorrhizal Fungus *Funneliformis mosseae*. *Plant and Cell Physiology*, 58(10), pp.1689-1699.
- Ramos, M., 2016.** Contribuição para a conservação sustentada vel de ecossistemas dunares invadidos por *Acacia longifolia*. MSc dissertation. University of Lisbon.
- Reyes-Torres, M., Oviedo-Ocaña, E.R., Dominguez, I., Komilis, D. and Sánchez, A., 2018.** A systematic review on the composting of green waste: Feedstock quality and optimization strategies. *Waste management*.
- Ribas-Agustí, A., Seda, M., Sarraga, C., Montero, J.I., Castellari, M. and Muñoz, P., 2017.** Municipal solid waste composting: Application as a tomato fertilizer and its effect on crop yield, fruit quality and phenolic content. *Renewable Agriculture and Food Systems*, 32(4), pp.358-365.
- Röös, E., Mie, A., Wivstad, M., Salomon, E., Johansson, B., Gunnarsson, S., Wallenbeck, A., Hoffmann, R., Nilsson, U., Sundberg, C. and Watson, C.A., 2018.** Risks and opportunities of increasing yields in organic farming. A review. *Agronomy for Sustainable Development*, 38(2), p.14.
- Satterthwaite, D., McGranahan, G. and Tacoli, C., 2010.** Urbanization and its implications for food and farming. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365(1554), pp.2809-2820.
- Seto, K.C. and Ramankutty, N., 2016.** Hidden linkages between urbanization and food systems. *Science*, 352(6288), pp.943-945.
- Skevas, T., Fevreiro, P. and Wesseler, J., 2010.** Co-existence regulations and agriculture production: A case study of five Bt maize producers in Portugal. *Ecological Economics*, 69(12), pp.2402-2408.

Tóth, G., Hermann, T., Da Silva, M.R. and Montanarella, L., 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. *Environment International*, 88, pp.299-309.

Tsvetkov, I., Atanasov, A., Vlahova, M., Carlier, L., Christov, N., Lefort, F., Rusanov, K., Badjakov, I., Dincheva, I., Tchamitchian, M. and Rakleova, G., 2018. Plant organic farming research—current status and opportunities for future development. *Biotechnology & Biotechnological Equipment*, 32(2), pp.241-260.

UNE-EN ISO/IEC 17025., 2018. <https://www.iso.org/obp/ui/#iso:std:iso-iec:17025:ed-3:v1:en>.

United Nations Human Settlements Programme., 2010. State of the World's Cities 2010/11: Cities for All: Bridging the Urban Divide. Earthscan, London, United Kingdom

Valorsul., 2018. <http://www.valorsul.pt/pt/seccao/areas-de-negocio/produtos/ricaterra-corretivo-agricola-organico/>

van Bueren, E.L., Jones, S.S., Tamm, L., Murphy, K.M., Myers, J.R., Leifert, C. and Messmer, M.M., 2011. The need to breed crop varieties suitable for organic farming, using wheat, tomato and broccoli as examples: a review. *NJAS-Wageningen Journal of Life Sciences*, 58(3), pp.193-205.

Wei, Y., Li, J., Shi, D., Liu, G., Zhao, Y. and Shi-maoka, T., 2017. Environmental challenges impeding the composting of biodegradable municipal solid waste: A critical review. *Resources, Conservation and Recycling*, 122, pp.51-65.

Chapter 6

General Discussion

6.1 Rhizosphere-litter-SOM interactions during the invasion process

Plant invasions are a fundamental driver of global change (Simberloff *et al.*, 2013). They are not only in themselves a major disruptive element for ecosystems worldwide, but also serve as a point of interaction, connecting the C, N and P (among many other) cycles. Furthermore, plants are the major primary producers of any terrestrial ecosystem, thus if, as is the case in *Acacia spp.* invasions, a diverse plant assembly is replaced by a plant monoculture, the effect necessarily needs to be profound (Mollot *et al.*, 2017). Additionally, invasive plants often benefit from human interference, while native ecosystems exhibit negative responses (Lilley and Vellend, 2009), thus ecosystems dominated by human presence are overrepresented as invaded systems on a global scale, which is especially the case for *Acacia* species (Gallien *et al.*, 2019). Humans also directly alter other important biogeochemical cycles, so this calls for a holistic view of plant invasion, which must necessarily include the nutrient pools and fluxes of ecosystems (Hulme *et al.*, 2009), especially if any one (or several) of the nutrients are limiting for ecosystem functioning, as is often the case in Mediterranean ecosystems (Ulm *et al.*, 2017).

6.1.1 Litter decomposition and its effects on SOM buildup

As shown in the first part of this work (chapters 2 and 3), invasive *Acacia* species are prolific ecosystem engineers, greatly increasing organic matter pools (**figure 2.2**, **table 3.1**) in

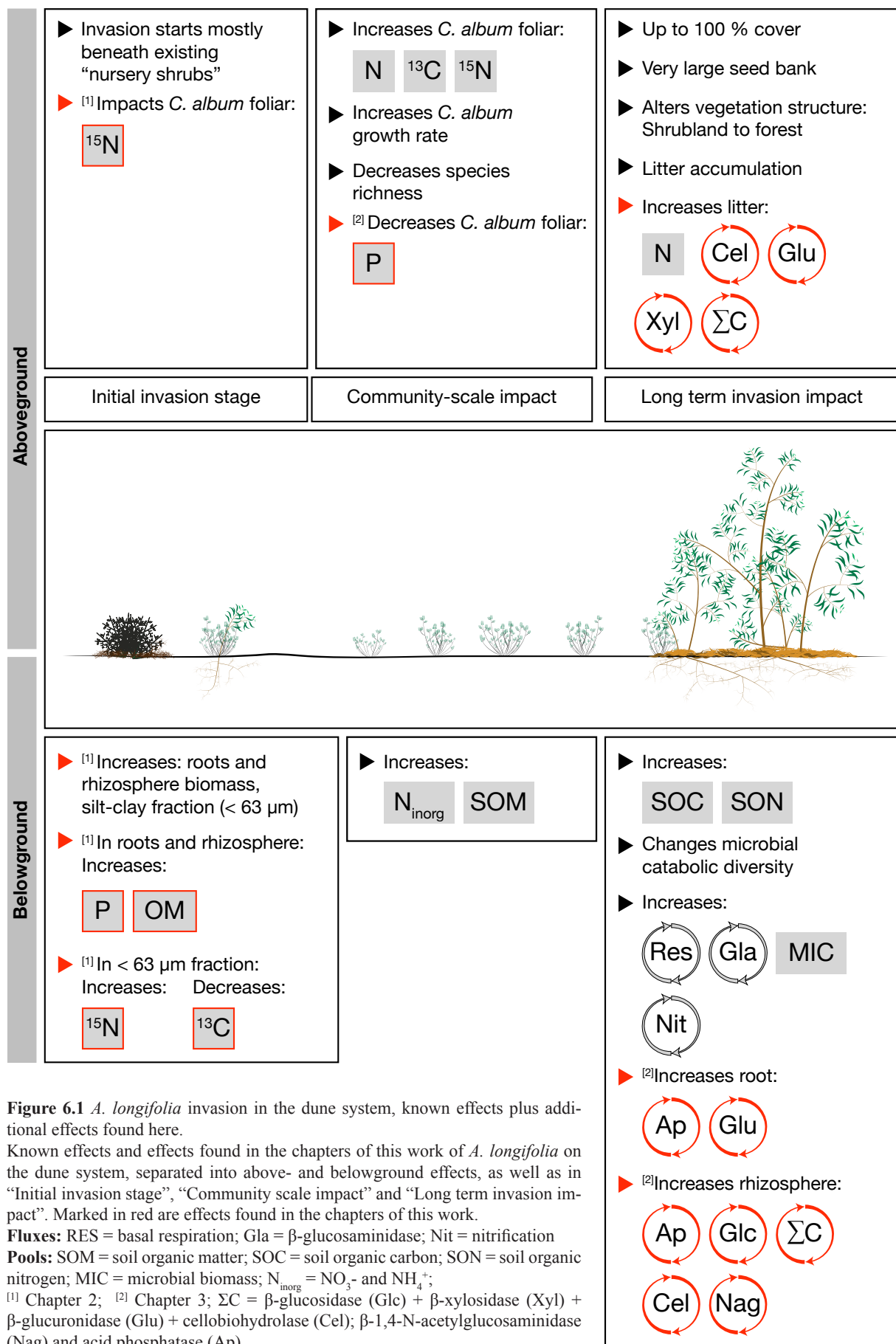
various soil compartments (**figure 6.1**). These C pools are not fixed, but rather in constant flux as indicated by the elevated potential extracellular enzyme activity (EEA) values found in most of the invaders degrading tissues when compared with native soil organic matter (SOM) pools (**table 3.3**). Increased potential organic matter (OM) turnover likely induces a situation of high C availability for local microbial communities, as indicated in chapter 3 by higher potential C turnover capacities in the invasives' rhizosphere, roots and litter. However, while EEA values were in general higher in the soil compartments of the invasive legume, the pattern of enzymatic activity differs: The invasives' litter exhibited only higher C-related EEA (**table 3.3**), while in its rhizosphere C, N and P-related EEA were all increased, compared to its native counterpart. This is in line with a recent meta-analysis of invasive plant species' effects on soil functioning via distinct litter and rhizosphere pathways (Zhang *et al.*, 2019). In their analysis, the authors found that is likely that invasive litter quantity and quality increases C availability and decomposer abundance, which would connect well to the higher C-related EEA observed here. Increased litter abundance underneath *A. longifolia* was reported repeatedly (Marchante *et al.* 2008b, Hellmann *et al.* 2011), however, this was not found in the work presented here (chapter 3). While seemingly contradictory, less observable litter could be related to higher turnover levels underneath the invader, which impede the buildup of lit-

ter through rapid degradation. This would also explain the relatively high C:N levels found (**table 3.1**), which is unusual for invasive species litter (Lee *et al.*, 2017). As a result of rapid litter turnover easily available C might get depleted by the high C-related EEA (**table 3.3**), leaving behind more recalcitrant OM. Indeed, this situation was found in a recent analysis comparing native litter with *A. longifolia* litter, with the latter exhibiting increasingly slower degradation rates along time despite its high N concentrations (Marchante *et al.*, 2019). While this degradation could lead to the accumulation of a recalcitrant litter layer, leached labile C in turn can be the precursor for micro-aggregate stable OM in sandy soils (Puttaso *et al.*, 2013), which is in accordance with the changes observed in the silt-clay SOM fraction (**figure 2.2**). This stabilized SOM in the silt-clay fraction is therefore most probably of microbial origin, and the flow and residence times of C and N in this compartment are related to nutrient availability and original substrate quality (Cyle *et al.*, 2016). Thus, if enough nutrients are available for microbial degradation of the litter layer, both the labile as well as the more recalcitrant OM pools can help to increase stable SOM (Cotrufo *et al.*, 2015), potentially delivering other benefits, such as nutrient and water retention. These effects are also positive for the native *C. album* plants, as indicated by their increased foliar N (**figure 3.2**). However, crucially, buildup of stable SOM occurs only very close to the invasives' own living biomass, for example next to small plants underneath the nursery shrubs (**figure 2.2**) as well the invasives' rhizosphere (**table 3.3**), therefore it can be assumed that the any positive effects feed back far stronger

on the invader itself than the native plants.

6.1.2 Rhizosphere: Contributing the nutrients for SOM degradation?

The rhizosphere is known to be an important hot spot of microbial activity, which is tightly connected to the input of labile C as well (Kuzyakov and Blagodatskaya, 2015). *A. longifolia* exhibits generally high growth rates, thus labile C input into its rhizosphere is likely, and additionally it has a strong capacity to fix N via its multiple symbiosis (Rodríguez-Echeverría *et al.*, 2007). The availability of C and N could explain the high EEA values in the rhizosphere (**table 3.3**), which further increase nutrient release, potentially through positive feedback loops between the rhizosphere and the litter layer (Subke *et al.*, 2004). Furthermore, root exudates in the rhizosphere as well as the root mass itself may contribute to the buildup of stable SOM, even if not in direct proximity to the rhizosphere (Angst *et al.*, 2016), which was indicated here by the strong association between belowground OM pools and the characteristics of the silt-clay fraction (**table 2.3**). Apart from the rhizosphere values themselves, also proxies, such as increased root N, indicate greater potential microbial C storage (Carrillo *et al.*, 2014). If this C ends up as microbial biomass in the rhizosphere, it is likely to be of fungal origin, as most invasive species have a negative impact on bacterial populations (Zhang *et al.*, 2019). This would also fit to the isotopic C signature of the silt-clay fraction found here, which was more depleted underneath the invasive (**table 2.2**) and potentially is a signal of higher fungal biomass (Kohl *et al.*, 2015). While fungal biomass was not measured here, its presence would also fit to the observed N transfer to the native vegeta-



tion, as it was observed that fungal networks potentially play an important role in this process (Teste *et al.*, 2015).

6.1.3 Belowground OM dynamics and the impact on N and P of the surrounding vegetation

Both N and C are being fixed from the atmosphere and are thus potentially limitless, while other nutrients, such as P, can potentially be depleted by plant growth along time, the so-called “sink-driven P depletion” (Vitousek *et al.*, 2010). Symbiotic N fixation is only very rarely constrained by P availability (Augusto, *et al.*, 2013) thus the invasion of a woody legume, such as *A. longifolia*, potentially induces a worsening of P availability in an ecosystem already low in nutrients. As the available P gets taken up, P pools shift to the biotic compartment (**figure 2.2**). This shift is correlated with *A. longifolia* foliar P, but not with *C. album* foliar P (**figure 2.3**), indicating the invasive species benefits more from the available P than the native. On the contrary, diminishing P is correlated with higher foliar N in *C. album*, an effect extending out into the surrounding vegetation (**figure 3.4**), potentially indicating higher systemic N availability coupled with a decrease in P. The impact of the legumes on the surrounding vegetation is also related to the legume root N concentrations (**figure 3.5**), which is a proxy of microbial activity in the rhizosphere (Carrillo *et al.*, 2014). In this compartment, C and N are readily available, while P is scarce, inducing competition between native and invasive plants for available P. Legume species can create “islands of N fertility” in N-limited ecosystems, which is also often well correlated with higher P avail-

ability (Rodríguez *et al.*, 2011). While no differences in available P were found between the native and the invasive legume (**table 3.2**), potentially this is also the case here, as indicated by the increased Ap values (**table 3.3**) underneath the invasive canopy. However, the native vegetation does not seem to benefit from this P, adding *A. longifolia* to other *Aca-**cia* species that are known to be able to shift N and P co-limited systems to a stronger P limitation (Sitters *et al.* 2013). A potential explanation for the N/P imbalance induced might be related to the differential movement of N and P in the soil. While inorganic N, such as NH_4^+ and NO_3^- have been shown to be distributed around plant canopies in a sandy soil in a spatially explicit manner, P does not (Rodríguez *et al.*, 2009). Similar to observations in other ecosystems (e.g. Xia *et al.*, 2015), fine-scale soil nutrient heterogeneity might be biotic driven and tightly connected to litter and root layers of the species observed, thus constraining nutrient availability in close proximity of the invader. A biotic agent that might be responsible for the nutrient distribution into the surrounding vegetation could be ectomycorrhizal fungi, as they are important for both N and P acquisition and *A. longifolia* is likely tapping into already existing ectomycorrhizal (ECM) networks (Carvalho *et al.*, 2018). The primary dunes exhibit extremely low soil nutrient concentrations, therefore it is probable that all plants both scavenge (through root proliferation) as well as mine (through symbiotic fungi) for P (Lambers *et al.*, 2008). An indication for the necessity of fungal mining of the soil for P are that most belowground OM exhibits C:P values far over 300 (**table 3.1**), which indicates strong P immobilization

(Souza-Alonso *et al.*, 2015). Thus, if mining is of high local importance, the increased availability of C from OM input, together with an abundance of available N, could drive N and P dislocation through the extended ECM hyphal network. ECM fungi exhibit N and P values close to their host plants (Kranabetter *et al.*, 2019) and they decrease and increase their biomass rapidly upon nutrient and water availability (Teste *et al.*, 2016), thus it could be plausible that if a fungal *A. longifolia* partner scavenges for P underneath *C. album* (or vice versa), the N (or P, respectively) stays underneath the canopy in the form of dead fungal biomass with N:P ratios similar to its host species, therefore “moving” P and N between plant species. While this hypothesis was not tested underneath *C. album* canopies surrounding the legume plants, $\delta^{15}\text{N}$ signatures of the silt-clay fraction under recently invaded *C. album* plants (**table 2.2**) are indicative of a strong legume influence in this compartment and as discussed in point 6.1.1 this fraction is quite likely of microbial origin. The invasive’s capacity to maintain high growth rates and therefore C sequestration would give its fungal symbionts a clear advantage to continue scavenging, as C allocation comes at barely any cost to the host plant (Corrêa *et al.*, 2012). Thus, assuming this dynamic exchange of P and N between islands of fertility (below plant canopies) is occurring, the invasive will necessarily turn into a P sink and a N source over time, exacerbating an existing lack of soil P. Furthermore, due to its high phenotypic plasticity, its symbiotic promiscuity and the positive plant-soil feedback loops, it maintains P flux into its own biomass, while accumulating N and C underneath its canopy, thereby

engineering SOM islands and inducing the transition of a species-rich shrub ecosystem to a monoculture forest. These findings add to amounting evidence that invasive plants not only change ecosystem biodiversity, but also significantly impact plant-soil elemental composition and stoichiometry, especially in nutri-

6.2 Composting *A. longifolia* biomass: Mimicry of its soil impacts?

ent-poor environments (Sardans *et al.*, 2016). The effects described in chapter 6.1 are clearly detrimental to native ecosystems, as they disrupt the prevalent nutrient balance, destroy existing niches and fundamentally transform the invaded system, resulting in a monocultures of little ecological value. However, if mimicked in the right situation, some of the same principles leading to ecosystem degradation might be channelled to help restore already degraded ecosystems, for example exhausted agricultural soils. Using invasive woody legumes, especially the fast growing *Acacia spp.*, for agricultural purposes is very controversial (e.g. Low, 2012 vs. Kull and Tassin, 2012). However, it is not a new idea and practiced in various areas as a novel agroforestry system with success (e.g. Tassin *et al.*, 2012). In Portugal, these kind of systems are presently unthinkable, as *Acacia spp.* are generally recognized as invasive species and their eradication propagated by citizen science projects and public awareness campaigns (Marchante and Marchante, 2016). It is also clear, however, that total *Acacia spp.* eradication is presently impossible and that new forms of management are needed (Souza-Alonso *et al.*, 2017), as for example the valuation of *Acacia* biomass by local stakeholders. A potential solution to many challenges associated with *Acacia* biomass usage, such as phytotoxicity and seed dispersal, is to use its chipped biomass as feedstock for green waste compost (Brito *et al.*, 2013). By degrading the biomass in this manner, a putatively well suited soil amendment can be

produced, as shown earlier in pot-scale experiments (Brito *et al.*, 2015a) and here in a local scale agricultural setting in chapter 5. However, while some work has been done on characterizing the composting process itself (Brito *et al.*, 2013, 2015b), a detailed description of *Acacia* GWC effects in the soil is lacking, as is a test of *Acacia* GWC as a safe and useful agricultural soil amendment.

6.2.1 From natural degradation to green waste compost

The natural degradation process of *A. longifolia* biomass clearly leads to increased SOM levels, as was shown in many prior publications and confirmed as well in chapter 2 and 3 of the work presented here. That this OM remains stable without continuous input from ongoing plant growth, however, was only recently reported and is probably mainly connected with its high lignin content (Marchante *et al.*, 2019). This is in accordance with what was reported for the composted *A. longifolia* biomass (Brito *et al.*, 2013) and points to a potentially stable OM, which is recommendable for a soil amendment. In agricultural soils there are many beneficial effects known for increased C and SOM levels, among them higher nutrient and water holding capacity, as well as improved soil structure and overall soil fertility and crop growth (Lal, 2004). While these effects are enabling organic agricultural systems to reach high levels of productivity, with yields comparable to conventional agriculture (Reeve *et al.*, 2016), organic amend-

ments such as compost can also greatly benefit conventional agricultural systems with inorganic nutrient input (Chen *et al.*, 2018; Luo *et al.*, 2018). An important issue for the usage of organic soil amendments is the mineralization and the immobilization of N (Masunga *et al.*, 2016). The determining factors for the immobilization or mineralization of N are the C:N ratio and degradability of the original biomass and the soil microbial community (Bengtsson, *et al.*, 2003). *A. longifolia* compost exhibited C:N ratios higher than 30 (point 4.3.1) and thus above the empirical threshold (20 - 25) of N mineralization vs. immobilization (Ceglie and Abdelrahman, 2014). In accordance with this observation, a clear NH_4^+ and NO_3^- immobilization was found in the experiment conducted here (chapter 4) both for pure compost (CO) treatments as well as compost/litter treatments (CL) in comparison to soil without amendment (**figure 4.1**, before NH_4^+ addition at day 73). While this immobilization can be problematic in certain situations and time scales, as microorganisms compete with plants for available N (Inselsbacher *et al.*, 2010), this process can also have various benefits, such as decreasing N_2O emissions as well as net N loss through leaching (Reichel *et al.*, 2018). It was found that GWC is especially well suited for that purpose by both decreasing N_2O emissions and N immobilization (Vaughan *et al.*, 2011). In the experiment conducted here (chapter 4), NH_4^+ was added after initial NH_4^+ was used up to mimic fertilization. This NH_4^+ rapidly got nitrified in all cases (**figure 4.1**, **figure 4.4**), however, NO_3^- accumulation was high in the sandy agricultural soil, which is in line with other observations showing no, or very little microbial

NO_3^- immobilization by soil microorganisms (Shi and Norton, 2000). However, increasing C availability can increase microbial N immobilization (Burger and Jackson, 2003) and indeed both GWC treatments exhibited lower net nitrification (**figure 4.1**), probably by immobilizing large parts of NH_4^+ in the microbial biomass before nitrification could take place. Interestingly, the nitrification post NH_4^+ addition correlated well with P availability (**figure 4.2**, **figure 4.5**), which was especially strong in the CL treatment. As a soil amendment in a conventional agricultural situation with inorganic N fertilization this could be an important property, as CL application increases P cycling and thus availability as well as N retention, while generally increasing soil health, as measured by an increment in V_{max} as well as K_m EEA values (**figure 4.3**). This increase in soil EEA was found in other *Acacia* spp. compost types (Tejada *et al.*, 2014) and increased N, C and P cycling upon addition of *A. longifolia* GWC is in accordance with observations from applications of other organic amendments, putatively leading to more plant available nutrients (Luo *et al.*, 2018). These findings are important as they add evidence of potential benefits of GWC amendments in agricultural settings, however, they might also be interesting from an ecological perspective: Final litter quantity was low in the CL treatments, (10 % litter in the compost and therefore 0.2 % in the soil mix observed in chapter 4), but nevertheless a clear difference of the CL treatment vs. the CO treatment was observed (**figure 4.1**, **figure 4.2**, **figure 4.4**). This implies that there must be a biotic effect of the *Acacia* litter, which is independent of its biophysical attributes and survives the hard con-

ditions of the composting process. It is known that many microorganisms, such as pathogens, may survive the composting process (Jones and Martin, 2003), thus it is conceivable that for example phosphorus solubilizing bacteria or certain fungi survived the composting. In any case, the main difference of the CO and the CL treatment is an increased nitrification in the latter, connected with a higher P availability. Taken together with observations from chapter 2 and 3, these results could help to explain the effects seen in the native ecosystems, as the litter biota increase N leaching and P mineralization underneath the canopy of the invasive species, therefore helping to drive the N/P imbalance observed. Summarized from an agricultural point of view, the addition of litter to the *A. longifolia* biomass did not jeopardize GWC quality, did not yield viable seeds and might even introduce interesting characteristics to the resulting soil amendment.

6.2.2 Living up to the reality test? An urban case study of *Acacia longifolia* green waste compost

Chapter 4 was more concerned with the addition of *A. longifolia* GWC in a conventional agricultural setting, however, GWC application should be seen in the wider context of an emerging change in the paradigm of agricultural practice and food security. Rapid expansion of most cities intensifies peri-urban agricultural land loss (Seto and Ramankutty, 2016) and increases the distance between sites of food production and consumption, therefore increasing greenhouse gas emissions (GHG) and food waste (Bloem and de Pee, 2017). This food waste, together with organic matter from urban green spaces con-

stitutes the largest source of municipal solid waste (Reyes-Torres *et al.*, 2018), which ends up in landfills, further diminishing nearby agricultural area (Adhikari *et al.*, 2010). Additionally, landfills are invasive species hot-spots, and *Acacia spp.* are among the species often found to spread in and around landfill sites and rubbish dumps (Plaza *et al.*, 2018). Thus, producing GWC from invasive *Acacia spp.* and using it in urban and peri-urban agriculture follows recent UN recommendations of working with local waste streams to foster circular economies and enable higher food sovereignty of urban populations (UN Environment, 2019). If, as shown in chapter 5, only locally sourced biomass is used without the application of inorganic fertilizer and if reusable (open-pollinated) crop varieties are used, the (urban) agricultural exploration is essentially independent of any outside inputs, thus paving the way for more sustainable local food networks (Sondermann *et al.*, 2016). To that end, the yield values found in the experiment reported in chapter 5 (**figure 5.1**) make clear that in an organic agricultural setting *A. longifolia* GWC can at least in parts substitute nutrient-rich compost, like municipal compost. What is important to note in this context is that it is nearly impossible to achieve these compost loads in a normal agricultural situation covering hectares of cropping fields. The quantities used as soil amendments in chapter 5 were more similar to their usage as the sole substrate (ca. 50 % , e.g. Brito *et al.*, 2015 a), rather than a soil amendment, which would be closer to 2 % as described in chapter 4 (e.g. Tejada *et al.*, 2014). Indeed, the SOC levels achieved in this experiment are already in the upper range, or even surpassing (**table 5.1**),

the so-called SOC stabilization level of 7.8 % (O’rourke *et al.*, 2015). However, considering that SOC might stabilize only in time scales of 20 to 60 years in agricultural soils (West and Post, 2002), it is unlikely that the stabilization point is already reached. Thus, as invasive species exhibit continuous rapid growth and are still introduced regularly in urban habitats (Potgieter *et al.*, 2019), this amendment regime seems to be feasible to maintain over longer time scales. Even with these high compost application levels, there were no negative consequences found on maize kernel nutritional status by *A. longifolia* GWC application (**table 5.2**). Furthermore, from a nutritional standpoint, the plant background seemed to be far more important than the soil amendment used, as all data shows clear variety dependent effects that were independent of soil treatments (**table 5.2, figure 5.2, figure 5.4, figure 5.5**). Especially in terms of heavy metal pollution, this observation is of great importance, as GWC application can in some cases lead to higher heavy metal leaching from already contaminated soils (Beesley and Dickinson, 2010), thus potentially making heavy metals more available to plants. In the experiment conducted here, the heavy metal concentration was low in general and was further diluted by the addition of *A. longifolia* compost (**table 5.2**). This is accordance with composts made from heavy metal loaded sewage sludge with *Acacia dealbata* biomass (Tejada *et al.*, 2014), which showed an improvement of soil health. While this could be connected solely to the GWC addition, it might also be related to the interaction of the OPV with soil microbial activity, like arbuscular mycorrhizal fungi, which can selectively exclude heavy

metals before plant uptake (Ramírez-Flores *et al.*, 2017). Higher mycorrhizal colonization is likely, as OPVs of maize are generally more prone to symbiotic relationships (Hess *et al.*, 2005), especially darker OPVs (as the variety used here) under medium level P availability (Sangabriel-Conde *et al.*, 2015). The resulting low levels of heavy metal pollution in the maize kernels, together with accumulation of micronutrients important for human health, such as zinc (**table 5.2**), makes the OPV used here a good choice for urban organic agriculture. Thus, from the perspective of using *A. longifolia* compost in agricultural settings, the benefits clearly outweigh known risks of compost application, such as heavy metal pollution and food chain contamination (Sharma *et al.* 2019). As, relatively speaking, urbanization is especially strong in Mediterranean areas (Valentini *et al.*, 2017), the prospect of using *A. longifolia* as a soil amendment is very promising in this circumstances. Indeed, adding to earlier findings about compost application in urban soils, which described application rates of compost up to 1/3 of the soil volume (Cogger, 2005), even very high application rates (ca. 50% v/v) were found to be beneficial for maize growth, thus opening up the possibility to increase SOM, soil quality and crop produc-

6.3 Conclusion

tion at the same time.

This work was concerned with the effects of the invasive leguminous *A. longifolia* on soil biomass and nutrient turnover, both *in situ* as well as when using its composted biomass as an agricultural soil amendment.

Many indicators found in chapters 2 and 3 point to increased soil N and P cycling as well as a high nutrient use efficiency as the basis for the remarkable growth rates of the invasive and thus its impact on the surrounding vegetation. Even in early invasion stages it impacts soil functioning, for example by increasing the silt-clay fraction of the sandy dune soil, with potentially beneficial effects. In both case studies presented here, there was no indication that native species do not benefit from these soil changes. Rather, it could be the localized nature of soil impacts (close to the invasives' own roots) that are more beneficial to the invader than the native plants, as well as secondary effects, such as increased competition for phosphorus, that are detrimental to the native ecosystem in the long run.

In accordance with these observations, the application of composted *A. longifolia* biomass showed no detrimental effect on maize growth. On the contrary, both laboratory scale soil manipulation with this compost as well as the addition of large amounts of compost in an urban agricultural setting increased soil organic matter concentrations and nutrient turnover, while in the latter case additionally benefiting maize growth. However, these findings now need to be corroborated in a larger field scale trial and the effect of *A. longifolia* compost needs to be evaluated for several growing

seasons to examine if there could be a buildup of potentially phytotoxic compounds or other detrimental effects. Furthermore, it is imperative to construct a simple and precise tool, such as an allometric biomass model, to estimate *A. longifolia* biomass in the field as to give local stakeholders the capability to assess if cutting, chipping, composting and subsequent application in the field is economically viable for the benefits it brings.

Lastly, from a conservation and fundamental research perspective, there is still the need to understand if the effects of *A. longifolia* (and to a certain extent *S. spectabilis*) found on *C. album* are similar for other dune species and in other regions of the globe. This assessment would give insight into the potential eutrophication of the dune system by the woody legume invader, adding to evidence of long-lasting effects on soil level even after aboveground biomass is removed. Also, as the dunes are very oligotrophic in nature, these observations could also help to answer some more fundamental questions about plant co-existence: up to what point the species found here do rely on each other, for example through mycorrhizal networks, which might be manipulated by plant invasion to change ecosystem function-

ing.

6.4 References

- Adhikari, B.K., Trémier, A., Martinez, J. and Barrington, S., 2010.** Home and community composting for on-site treatment of urban organic waste: perspective for Europe and Canada. *Waste Management & Research*, 28(11), pp.1039-1053.
- Angst, G., Kögel-Knabner, I., Kirfel, K., Hertel, D. and Mueller, C.W., 2016.** Spatial distribution and chemical composition of soil organic matter fractions in rhizosphere and non-rhizosphere soil under European beech (*Fagus sylvatica* L.). *Geoderma*, 264, pp.179-187.
- Augusto, L., Delerue, F., Gallet-Budynek, A. and Achat, D.L., 2013.** Global assessment of limitation to symbiotic nitrogen fixation by phosphorus availability in terrestrial ecosystems using a meta-analysis approach. *Global Biogeochemical Cycles*, 27(3), pp.804-815.
- Beesley, L. and Dickinson, N., 2010.** Carbon and trace element mobility in an urban soil amended with green waste compost. *Journal of Soils and Sediments*, 10(2), pp.215-222.
- Bengtsson, G., Bengtson, P. and Månsson, K.F., 2003.** Gross nitrogen mineralization-, immobilization-, and nitrification rates as a function of soil C/N ratio and microbial activity. *Soil Biology and Biochemistry*, 35(1), pp.143-154.
- Bloem, S. and de Pee, S., 2017.** Developing approaches to achieve adequate nutrition among urban populations requires an understanding of urban development. *Global Food Security*, 12, pp.80-88.
- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S., 2013.** Composting for management and resource recovery of invasive *Acacia* species. *Waste Management & Research*, 31(11), pp.1125-1132.
- Brito, L.M., Mourão, I., Coutinho, J. and Smith, S.R., 2015. (a)** Co-composting of invasive *Acacia longifolia* with pine bark for horticultural use. *Environmental technology*, 36(13), pp.1632-1642.
- Brito, L.M., Reis, M., Mourão, I. and Coutinho, J., 2015. (b)** Use of acacia waste compost as an alternative component for horticultural substrates. *Communications in Soil Science and Plant Analysis*, 46(14), pp.1814-1826.
- Burger, M. and Jackson, L.E., 2003.** Microbial immobilization of ammonium and nitrate in relation to ammonification and nitrification rates in organic and conventional cropping systems. *Soil Biology and Biochemistry*, 35(1), pp.29-36.
- Carrillo, Y., Dijkstra, F.A., Pendall, E., LeCain, D. and Tucker, C., 2014.** Plant rhizosphere influence on microbial C metabolism: the role of elevated CO₂, N availability and root stoichiometry. *Biogeochemistry*, 117(2-3), pp.229-240.
- Carvalho, P., Martins, R., Portugal, A. and Gonçalves, M.T., 2018.** Do mycorrhizal fungi create below-ground links between native plants and *Acacia longifolia*? A case study in a coastal maritime pine forest in Portugal. *Web Ecology*, 18(1), pp.105-114.
- Ceglie, F.G. and Abdelrahman, H.M., 2014.** Ecological intensification through nutrients recycling and composting in organic farming. In: *Composting for Sustainable Agriculture*. Springer, Switzerland. pp. 1-22
- Chen, Y., Camps-Arbestain, M., Shen, Q., Singh, B. and Cayuela, M.L., 2018.** The long-term role of organic amendments in building soil nutrient fertility: a meta-analysis and review. *Nutrient cycling in agroecosystems*, 111(2-3), pp.103-125.
- Cogger, C.G., 2005.** Potential compost benefits for restoration of soils disturbed by urban development. *Compost science & utilization*, 13(4), pp.243-251.
- Corrêa, A., Gurevitch, J., Martins-Loução, M.A. and Cruz, C., 2012.** C allocation to the fungus is not a cost to the plant in ectomycorrhizae. *Oikos*, 121(3), pp.449-463.
- Cotrufo, M.F., Soong, J.L., Horton, A.J., Campbell, E.E., Haddix, M.L., Wall, D.H. and Parton, W.J., 2015.** Formation of soil organic matter via biochemical and physical pathways of litter mass loss. *Nature Geoscience*, 8(10), p.776.
- Cyle, K.T., Hill, N., Young, K., Jenkins, T., Hancock, D., Schroeder, P.A. and Thompson, A., 2016.** Substrate quality influences organic matter accumulation in the soil silt and clay fraction. *Soil Biology and Biochemistry*, 103, pp.138-148.
- Eldridge, S.M., Chan, K.Y., Donovan, N.J., Saleh, F., Orr, L. and Barchia, I., 2018.** Agronomic and economic benefits of green-waste compost for peri-urban vegetable production: implications for food security. *Nutrient Cycling in Agroecosystems*, 111(2-3), pp.155-173.
- Gallien, L., Thornhill, A.H., Zurell, D., Miller J.T. and Richardson D.M., 2019.** Global predictors of al-

ien plant establishment success: combining niche and trait proxies. *Proceedings of the Royal Society B: Biological Sciences*, 286

Hellmann, C., Sutter, R., Rascher, K.G., Máguas, C., Correia, O. and Werner, C., 2011. Impact of an exotic N₂-fixing *Acacia* on composition and N status of a native Mediterranean community. *Acta Oecologica*, 37(1), pp.43-50.

Hess, J.L., Shiffler, A.K. and Jolley, V.D., 2005. Survey of mycorrhizal colonization in native, open-pollinated, and introduced hybrid maize in villages of Chiquimula, Guatemala. *Journal of Plant Nutrition*, 28(10), pp.1843-1852.

Hulme, P.E., Pyšek, P., Jarošík, V., Pergl, J., Schaffner, U. and Vila, M., 2013. Bias and error in understanding plant invasion impacts. *Trends in Ecology & Evolution*, 28(4), pp.212-218.

Inselsbacher, E., Umana, N.H.N., Stange, F.C., Gorfer, M., Schüller, E., Ripka, K., Zechmeister-Boltenstern, S., Hood-Novotny, R., Strauss, J. and Wanek, W., 2010. Short-term competition between crop plants and soil microbes for inorganic N fertilizer. *Soil Biology and Biochemistry*, 42(2), pp.360-372.

Jones, P. and Martin, M., 2003. A review of the literature on the occurrence and survival of pathogens of animals and humans in green compost. WRAP standards report. The Waste and Resources Action Programme, Oxon, United Kingdom.

Kohl, L., Laganière, J., Edwards, K.A., Billings, S.A., Morrill, P.L., Van Biesen, G. and Ziegler, S.E., 2015. Distinct fungal and bacterial $\delta^{13}\text{C}$ signatures as potential drivers of increasing $\delta^{13}\text{C}$ of soil organic matter with depth. *Biogeochemistry*, 124(1-3), pp.13-26.

Kull, C.A. and Tassin, J., 2012. Australian acacias: useful and (sometimes) weedy. *Biological invasions*, 14(11), pp.2229-2233.

Kranabetter, J.M., Harman-Denhoed, R. and Hawkins, B.J., 2019. Saprotrophic and ectomycorrhizal fungal sporocarp stoichiometry (C: N: P) across temperate rainforests as evidence of shared nutrient constraints among symbionts. *New Phytologist*, 221(1), pp.482-492.

Lambers, H., Raven, J.A., Shaver, G.R. and Smith, S.E., 2008. Plant nutrient-acquisition strategies change with soil age. *Trends in ecology & evolution*, 23(2), pp.95-103.

Lal, R., 2004. Soil carbon sequestration impacts on global climate change and food security. *science*, 304(5677), pp.1623-1627.

Lee, M.R., Bernhardt, E.S., van Bodegom, P.M., Cornelissen, J.H.C., Kattge, J., Laughlin, D.C., Ninemets, Ü., Peñuelas, J., Reich, P.B., Yguel, B. and Wright, J.P., 2017. Invasive species' leaf traits and

dissimilarity from natives shape their impact on nitrogen cycling: a meta-analysis. *New Phytologist*, 213(1), pp.128-139.

Lilley, P.L. and Vellend, M., 2009. Negative native–exotic diversity relationship in oak savannas explained by human influence and climate. *Oikos*, 118(9), pp.1373-1382.

Low, T., 2012. In denial about dangerous aid. *Biological Invasions*, 14(11), pp.2235-2236.

Luo, G., Li, L., Friman, V.P., Guo, J., Guo, S., Shen, Q. and Ling, N., 2018. Organic amendments increase crop yields by improving microbe-mediated soil functioning of agroecosystems: A meta-analysis. *Soil Biology and Biochemistry*, 124, pp.105-115.

Marchante, E., Kjeller, A., Struwe, S. and Freitas, H., 2008. (b) Short-and long-term impacts of *Acacia longifolia* invasion on the belowground processes of a Mediterranean coastal dune ecosystem. *Applied Soil Ecology*, 40(2), pp.210-217.

Marchante, E. and Marchante, H., 2016. Engaging society to fight invasive alien plants in Portugal—One of the main threats to biodiversity. In *Biodiversity and Education for Sustainable Development* (pp. 107-122). Springer. Switzerland.

Marchante, E., Marchante, H., Freitas, H., Kjeller, A. and Struwe, S., 2019. Decomposition of an N-fixing invasive plant compared with a native species: Consequences for ecosystem. *Applied Soil Ecology*.

Masunga, R.H., Uzokwe, V.N., Mlay, P.D., Odeh, I., Singh, A., Buchan, D. and De Neve, S., 2016. Nitrogen mineralization dynamics of different valuable organic amendments commonly used in agriculture. *Applied Soil Ecology*, 101, pp.185-193.

Mollot, G., Pantel, J.H. and Romanuk, T.N., 2017. The effects of invasive species on the decline in species richness: a global meta-analysis. *Advances in Ecological Research*, 56, pp. 61-83.

O'rourke, S.M., Angers, D.A., Holden, N.M. and McBratney, A.B., 2015. Soil organic carbon across scales. *Global change biology*, 21(10), pp.3561-3574.

Plaza, P.I., Speziale, K.L. and Lambertucci, S.A., 2018. Rubbish dumps as invasive plant epicentres. *Biological Invasions*, 20(9), pp.2277-2283.

Potgieter, L.J., Gaertner, M., O'Farrell, P.J. and Richardson, D.M., 2019. Perceptions of impact: invasive alien plants in the urban environment. *Journal of environmental management*, 229, pp.76-87.

Puttaso, A., Vityakon, P., Rasche, F., Saenjan, P.,

- Treloges, V. and Cadisch, G., 2013.** Does organic residue quality influence carbon retention in a tropical sandy soil?. *Soil Science Society of America Journal*, 77(3), pp.1001-1011.
- Ramírez-Flores, M.R., Rellán-Álvarez, R., Wozniak, B., Gebreselassie, M.N., Jakobsen, I., Olalde-Portugal, V., Baxter, I., Paszkowski, U. and Sawers, R.J., 2017.** Co-ordinated Changes in the Accumulation of Metal Ions in Maize (*Zea mays ssp. mays* L.) in Response to Inoculation with the Arbuscular Mycorrhizal Fungus *Funneliformis mosseae*. *Plant and Cell Physiology*, 58(10), pp.1689-1699.
- Reeve, J.R., Hoagland, L.A., Villalba, J.J., Carr, P.M., Atucha, A., Cambardella, C., Davis, D.R. and Delate, K., 2016.** Organic farming, soil health, and food quality: considering possible links. In: *Advances in Agronomy*, 137, pp. 319-367.
- Reichel, R., Wei, J., Islam, M.S., Schmid, C., Wisel, H., Schröder, P., Schloter, M. and Brüggemann, N., 2018.** Potential of wheat straw, spruce sawdust, and lignin as high organic carbon soil amendments to improve agricultural nitrogen retention capacity: an incubation study. *Frontiers in plant science*, 9, p.900.
- Reyes-Torres, M., Oviedo-Ocaña, E.R., Dominguez, I., Komilis, D. and Sánchez, A., 2018.** A systematic review on the composting of green waste: Feedstock quality and optimization strategies. *Waste management*.
- Rodríguez, A., Durán, J., Fernández-Palacios, J.M. and Gallardo, A., 2009.** Spatial pattern and scale of soil N and P fractions under the influence of a leguminous shrub in a *Pinus canariensis* forest. *Geoderma*, 151(3-4), pp.303-310.
- Rodríguez, A., Durán, J., Covelo, F., Fernández-Palacios, J.M. and Gallardo, A., 2011.** Spatial pattern and variability in soil N and P availability under the influence of two dominant species in a pine forest. *Plant and soil*, 345(1-2), pp.211-221.
- Rodríguez-Echeverría, S., Crisóstomo, J.A. and Freitas, H., 2007.** Genetic diversity of rhizobia associated with *Acacia longifolia* in two stages of invasion of coastal sand dunes. *Applied and environmental microbiology*, 73(15), pp.5066-5070.
- Sangabriel-Conde, W., Maldonado-Mendoza, I.E., Mancera-López, M.E., Cordero-Ramírez, J.D., Trejo-Aguilar, D. and Negrete-Yankelevich, S., 2015.** Glomeromycota associated with Mexican native maize landraces in Los Tuxtlas, Mexico. *Applied Soil Ecology*, 87, pp.63-71.
- Sardans, J., Bartrons, M., Margalef, O., Gargallo-Garriga, A., Janssens, I.A., Ciais, P., Obersteiner, M., Sigurdsson, B.D., Chen, H.Y. and Peñuelas, J., 2017.** Plant invasion is associated with higher plant-soil nutrient concentrations in nutrient-poor environments. *Global change biology*, 23(3), pp.1282-1291.
- Seto, K.C. and Ramankutty, N., 2016.** Hidden linkages between urbanization and food systems. *Science*, 352(6288), pp.943-945.
- Simberloff, D., Martin, J.L., Genovesi, P., Maris, V., Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M. and Pyšek, P., 2013.** Impacts of biological invasions: what's what and the way forward. *Trends in ecology & evolution*, 28(1), pp.58-66.
- Sitters, J., Edwards, P.J. and Venterink, H.O., 2013.** Increases of soil C, N, and P pools along an acacia tree density gradient and their effects on trees and grasses. *Ecosystems*, 16(2), pp.347-357.
- Sondermann, M., Morán, N. and Ioannou, B., 2016.** Grassroots gardening movements: towards cooperative forms of green urban development?. In: *Urban allotment gardens in Europe* (pp. 84-112). Routledge. United Kingdom
- Souza-Alonso, P., Guisande-Collazo, A. and González, L., 2015.** Gradualism in *Acacia dealbata* Link invasion: impact on soil chemistry and microbial community over a chronological sequence. *Soil Biology and Biochemistry*, 80, pp.315-323.
- Souza-Alonso, P., Rodríguez, J., González, L. and Lorenzo, P., 2017.** Here to stay. Recent advances and perspectives about *Acacia* invasion in Mediterranean areas. *Annals of Forest Science*, 74(3), p.55.
- Subke, J.A., Hahn, V., Battipaglia, G., Linder, S., Buchmann, N. and Cotrufo, M.F., 2004.** Feedback interactions between needle litter decomposition and rhizosphere activity. *Oecologia*, 139(4), pp.551-559.
- Tassin, J., Rangan, H. and Kull, C.A., 2012.** Hybrid improved tree fallows: harnessing invasive woody legumes for agroforestry. *Agroforestry systems*, 84(3), pp.417-428.
- Tejada, M., Gómez, I., Fernández-Boy, E. and Díaz, M.J., 2014.** Effects of sewage sludge and *Acacia dealbata* composts on soil biochemical and chemical properties. *Communications in soil science and plant analysis*, 45(5), pp.570-580.
- Teste, F.P., Veneklaas, E.J., Dixon, K.W. and Lambers, H., 2015.** Is nitrogen transfer among plants enhanced by contrasting nutrient-acquisition strategies?. *Plant, cell & environment*, 38(1), pp.50-60.
- Teste, F.P., Laliberté, E., Lambers, H., Auer, Y., Kramer, S. and Kandeler, E., 2016.** Mycorrhizal fungal biomass and scavenging declines in phosphorus-impooverished soils during ecosystem retrogression. *Soil Biology and Biochemistry*, 92, pp.119-132.
- Ulm, F., Gouveia, C., Dias, T. and Cruz, C., 2017.** N

fertilization in a Mediterranean ecosystem alters N and P turnover in soil, roots and the ectomycorrhizal community. *Soil Biology and Biochemistry*, 113, pp.60-70.

UN Environment., 2019. Global Environment Outlook – GEO-6: Healthy Planet, Healthy People. Nairobi.

Vaughan, S.M., Dalal, R.C., Harper, S.M. and Menzies, N.W., 2011. Effect of fresh green waste and green waste compost on mineral nitrogen, nitrous oxide and carbon dioxide from a Vertisol. *Waste Management*, 31(8), pp.1720-1728.

Vitousek, P.M., Porder, S., Houlton, B.Z. and Chadwick, O.A., 2010. Terrestrial phosphorus limitation: mechanisms, implications, and nitrogen–phosphorus interactions. *Ecological applications*, 20(1), pp.5-15.

Wardle, D.A., Aronson, J., Courchamp, F., Galil, B., García-Berthou, E., Pascal, M. and Pyšek, P., 2013. Impacts of biological invasions: what's what and the way forward. *Trends in Ecology & Evolution*, 28(1), pp.58-66.

West, T.O. and Post, W.M., 2002. Soil organic carbon sequestration rates by tillage and crop rotation. *Soil Science Society of America Journal*, 66(6), pp.1930-1946.

Xia, S.W., Chen, J., Schaefer, D. and Detto, M., 2015. Scale-dependent soil macronutrient heterogeneity reveals effects of litterfall in a tropical rainforest. *Plant and Soil*, 391(1-2), pp.51-61.

Zhang, P., Li, B., Wu, J. and Hu, S., 2019. Invasive plants differentially affect soil biota through litter and rhizosphere pathways: a meta-analysis. *Ecology letters*, 22(1), pp.200-210.